STUDIES ON PREPARATION AND USE OF BOVINE PEPSIN FROM ADULT CATTLE ABOMASA AS RENNET SUBSTITUTE FOR SMALL SCALE CHEESE PROCESSING

BY

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ABSTRACT

World-wide shortage of rennet has stimulated interest in the search for rennet substitutes. In order to overcome the scarcity and high price for rennet in Tanzania, studies on preparation of a rennet substitute from cattle abomasa were undertaken in three experiments

Experiment one dealt with investigations into the optimisation of pepsin extraction conditions i.e. on regions of abomasa, extraction of dry versus fresh material, drying methods, ionic strength, extraction pH, temperature, time, activation pH and clarification methods. Results showed that the fundic region, sundrying, 10% salt, extraction with HCL at pH 3 for 3 days, at 37° C, activation pH 2 for 30 min and clarification using $Na_2HPO_4.H_2O$ produce an extract with maximum milk clotting activity (MCA). Optimum conditions were applied to extract pepsin with dilute HCL, vinegar and lemon juice. Vinegar (VP) and HCL (HP) gave better yield, shelf-life, proteolytic activity, chymosin:pepsin ratio, microbial quality and formagraph characteristics than lemon juice. Concentrating VP extract lead to greater loss in MCA than for HP extract.

In experiment two, HP extract was purified by DEAE cellulose chromatography, gel filtration, mono Q and phenyl sepharose chromatography. Studies on the purified enzyme with regard to formagraph characteristics, molecular mass, sodium dodecylsulphate gel electrophoresis (SDS-PAGE) and N-terminal amino acid sequence showed that pepsin was homogenous with fold purification of 366, recovery rate of 32.7%,

Specific activity 3920 MCA (units/mg), molecular mass 34,500 Da. The N-terminus of purified pepsin contained no lysine and the MCA decreased with enzyme purity.

In experiment 3, HP and VP extracts were mixed with rennet at 0, 25, 50, 75, 100% and used for making three types of cheese. Renneting time (RT) for cheese milk, butterfat (%BF) and total nitrogen (%TN) in whey, chemical composition and microbial quality of cheese samples obtained at different storage time intervals, sensory evaluation at maturity and overall quality assessment were determined. RT and TN losses increased with level of pepsin; chemical composition of all cheese types were significantly different ($P<.001$). The pH in SUA Pastafilata and SUA Alpine and TN in Tilsiter cheese were not significantly different ($P>0.05$). Sensory evaluation was not significantly different ($P>0.05$) in smell, taste, appearance and bitterness, however, bitterness in SUA Pastafilata and appearance in SUA Alpine were significantly different ($P<0.05$); appearance in SUA Pastafilata and village Alpine were significant different ($P<0.01$). Bitterness, taste in SUA Alpine and bitterness in Tilsiter cheeses were highly significant different ($P<.001$). Overall quality assessment of cheese did not show significant differences ($P>0.05$). Therefore all enzyme combinations were suitable for all types of cheese implying that pepsin was an appropriate rennet substitute.

DECLARATION

I, Ruth Nnala Z. Ryoba, do hereby declare to the Senate of Sokoine University of Agriculture that the thesis presented here is my own original work and that to the best of my knowledge this work has not been submitted to any other University for a degree award.

Signature.....

Date.....23/11/1999

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DEDICATION

Dedicated to my father and mother

Andingenye and Hellen

To My Heavenly Father, His Son Jesus Christ and the Holy Spirit be glory power and praise. Behold, God is exalted by His power; who teaches like HIM?

Job 36:22.

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ABBREVIATIONS AND SYMBOLS

A_{30}	Amplitude after the gelling point (mm)
AD	Agar diffusion diameter
BF	Butterfat
Coli	Coliform bacteria
CR	Commercial calf rennet
CT	Clotting time
HCl	Hydrochloric acid
HP	Pepsin extracted using dilute HCl
K_{20}	Time to reach 20 mm from gelling point (min)
LP	Pepsin extracted using lemon juice
MCA	Milk clotting activity
MM	Molecular mass
OD	Optical density
Pasta	Pastafilata cheese
PU	Pepsin unit
R	Time until start of milk gelling (min)
RI	Ripening index
SPC	Standard plate count
STD	Standard
SUA	Sokoine University of Agriculture
TN	Total nitrogen
UF	Ultra filtration

VP	Pepsin extracted using commercial vinegar
WSN	Water soluble nitrogen
YM	Yeast and mould

CHAPTER 1

GENERAL INTRODUCTION

Total milk production in Tanzania is about 560 million liters per year. Due to low purchasing power in the areas with successful dairy development schemes, milk surpluses above local consumption are quickly attained forcing milk producers to look for distant markets (Scheinmann *et al.* 1992; Kurwijila and Henriksen, 1995). The surplus milk above the family's domestic processing and consumption needs and/or capacity is usually marketed as fresh milk if there is a marketing outlet (Ryoba and Kurwijila, 1990). Unfortunately during the rainy season, impassable roads make marketing of fresh milk almost impossible (Lohay, 1988). Thus there is a need to train farmers on how to produce milk of high quality and promote development of simple processing methods and preservation of products to absorb the fresh milk surpluses.

Cheesemaking and milk powder manufacturing are some of the most efficient ways used in many countries to preserve milk. In cheesemaking, 10 - 12 l of fresh milk are used to produce one kg of cheese which can be stored up to one year and is easy to transport to other parts of the country. Cheese processing has been successfully introduced in Kagera (De Wolf, 1990), Hai district (the Losaa Women Group) (Ulicky, 1989) and at Mulala village (Arumeru) (Kurwijila, 1990). Some commercial dairy farmers in Tanga, Njombe, Mbeya, Iringa, Arusha and Kilimanjaro process some fresh milk into cheese to enhance their income (Kurwijila, 1990 and Ministry of Agriculture and Livestock Divelopment (MALD, 1990).

A major problem facing cheesemaking enterprises in different regions of Tanzania is the lack of locally produced rennet, a coagulant necessary to produce any type of ripening cheese. Most producers rely on donations from abroad which is not a very sustainable way of developing a dairy industry. A good example was that facing the Losaa Women Group in Hai district who were stranded for lack of rennet after the termination of FAO support at the end of 1990 (Kurwijila, 1990). The small size of the cheese production industry in Tanzania makes the importation of even small quantities of rennet commercially difficult to justify and is rather uneconomical. Hence there are no local commercial suppliers of rennet.

Rennet is usually extracted from the 4th stomach of the young calf (normally called chymosin) when it is less than 4 weeks old. However, male calves are highly valued by farmers in Tanzania and hence it would not be practical to depend on calves for chymosin. The shortage of calves even in developed countries has led to use of rennet substitutes such as microbial proteinases, porcine pepsin, bovine pepsin and recently genetically recombined microbial rennet (Farmakalids, 1995; Dajnowiec *et al.*1997). Production of microbial coagulants needs a very high technical know how and requires sophisticated equipment and initial costs are extremely high (Webb *et al.* 1974). Porcine pepsin can not be accepted in some parts of Tanzania due to religious considerations. The only alternative is to use bovine pepsin which has been used successfully in other parts of the world (Green, 1972; Davide *et al.*1982). Chri Hansen's Laboratory produce a milk coagulant containing 50% bovine pepsin, 30% porcine pepsin and 20% calf rennet under the trade name B-P TM (Chri. Hansen laboratory Inc,1990). Bovine pepsin is extracted from adult cattle abomasa. Since in Tanzania

adult cattle are slaughtered daily in thousands, this could provide readily and abundant supply of pepsin.

There have been some studies at SUA Dairy Technology Laboratory on the extraction of bovine pepsin, evaluation of cheese made from pepsin and investigation on pepsin shelf life (Lyatuu, 1991; Ssekaalo, 1992). Very promising results were obtained and yield was found to be 17.6 g. crude pepsin extract per 100 g of the abomasal mucosa which can produce about 16 kg cheese (Wigenge, 1989; Mugenyi, 1990; Lyatuu, 1991). Since the study periods were too short and the results were few it was difficult to draw any solid conclusion which could be recommended.

The purpose of the present research was therefore to conduct a more detailed study on the extraction of bovine pepsin, using materials locally available in Tanzania i.e. lemon juice and vinegar in addition to dilute HCl, purify the crude extract using ion exchange chromatography so that the pure enzyme can be studied and characterised. The results of the study should enable the establishment of a local capability at SUA Dairy Technology which can be easily transferred to dairy processors or local laboratories in Tanzania.

Specific objectives

1. To develop a suitable method of preparing crude pepsin extract from adult cattle abomasa and recommend some of the local materials which can be used to extract and preserve crude pepsin.
2. To concentrate the extract preparation and determine its MCA
3. To purify the crude extract using ion exchange chromatography methods and characterize the purified enzyme.
4. To determine the suitability of the enzyme preparations in the manufacture of three common types of cheeses made in Tanzania.

Hypothesis: The study will seek to verify the following hypotheses:

Locally produced crude bovine pepsin extracts can effectively replace commercial rennet in cheesemaking without loss of quality of the product.

The crude extracts can be processed and purified into forms that can be easily stored without affecting their MCA.

CHAPTER 2

LITERATURE REVIEW

2.1 Role of rennet in cheese making

Calf rennet is the coagulating enzyme preparation traditionally used for cheesemaking in most parts of the world. It was the sole coagulant until the late 1950 (Phelan, 1977; Andren *et al.* 1981). Calf rennet is extracted from the abomasa of calves fed milk up to 30 days of age (Green, 1972; Phelan, 1977; Andrens *et al.* 1981, 1990; Peralta *et al.* 1985). Since the fifties there has been a large increase in cheese production and consumption and a simultaneous decrease in the amount of calf stomachs because an increasing number of calves were allowed to grow to maturity to increase the supply of meat protein thereby decreasing the supply of calf abomasa (Green, 1972; Sardinas, 1976; Davide *et al.* 1982; Peralta *et al.* 1985). In the US, cheese production almost doubled between 1960 and 1973 to 1.2 billion kg. During the same period, calves slaughtered for abomasa production decreased from 8 to 2 million kg annually due to calves being allowed to grow to greater slaughter weight (Phelan, 1977; Nelson, 1975; Sardinas, 1976). Therefore, in the past two decades, there has been a worldwide shortage of calf rennet which led to price rises and decreased availability of rennet. This stimulated the worldwide interest in the search for alternative coagulants or rennet substitutes. The search has been widespread and intensive (Table1) where plants, animals and microbial sources have been investigated (Sardinas, 1976; Andren, 1985, 1992; Ortz de Apodaca *et al.* 1994; Mohamed and O'Connor, 1996; Dajnowiec, 1997).

Table 1: Sources and Nomenclature of major proteases

Source	Enzyme	14 B-name*	Trade names
Plants			
Ficus carica	Ficin		
Pineapple	Bromelain		
Papaya	Papain		
Microbial	*Mucor miehei*		Rennilase (Novo)
	Acid Protease	EC 3.4 23.6	Halnilase (Chr. Hansen)
	(MMP)		Fromase (Wallerstein)
			Marzyme (Miles)
Mucor pussilus	*M. pusillus*		Emporase (Dairy land)
var lindt	Acid Protease (MPP)	EC 3.4 23.6	Meito (Meito sangyo)
			Noury (Vitex)
Endothia parasitica	*E. parasitica*		Sure curd
	Acid ptease	EC 3.4 23.6	Suparen pfizer
			(Cchy-max)
Animals:Ruminants	Chymosin	EC 2.4 23.4	Chymosin
Ruminants and pigs	Gastricsin-pepsin	EC 3.4 23.3	Pepsin I
			Parapepsin II
			Pepsin B
Ruminants,	Pepsin	Pepsin A	Pepsin II
Chicken		EC 3.4 23.1	Chicken pepsin Weismann
fish			Fish pepsin
pigs, ,			Porcine pepsin (Chr. Hansen)

(Phelan,1977; Foltmann, 1987; Broome and Hickey. 1990).

* International Units of Biochemical. EC is number of biochemical name

2.1.1 Rennet substitutes

a) Plant coagulants

Rennet substitutes from higher plants give some problems although the source is abundant. The enzymes besides having milk clotting ability, were strongly proteolytic. This resulted in extensive digestion of the curd with subsequent reduced yield, pasty body and formation of bitter flavour in the cheese (Whitaker, 1972; Webb *et al.* 1974; Phelan, 1977; Green, 1977). Furthermore crude preparations of plant derived enzymes contain some heavy metal ions which give enzyme activity inhibition (Whitaker, 1972 and Webb *et al.* 1974). They also contain high levels of phenolic compounds which normally combine with broken cells and inactivate the enzyme. Compared to animal enzymes, plant enzymes are more resistant to heat and oxidising agents (Davis, 1965; Mohamed and O'Connor, 1996; Asakura *et al.*1997). Verissimo *et al.* (1996) and Mohamed and O'Connor (1996) reported the use of *Cynara cardunlulus L.*a fresh flower and *Calotropis procera* aspartic proteinase in cheese making respectively.

b) Microbial coagulants

Production of rennet substitutes have also been made from microbes. Some microbial rennets have shown good activity whereas others gave similar problems as extracts from higher plants, (Webb *et al.* 1974; Farmakalids, 1995; Dajnowiec *et al.*1997). Bacterial enzymes such as protease extracted from *Streptococcus liquefaciens, Bacillus cereus* were also used as rennet substitutes (Brown *et al.* 1988). Enzymes from fungi such as *Aspergillus glanucus, A. nidulans* and *Cladosporium herbarum* reported for instance by Webb *et al.* (1974) and Ortiz de Apodaca (1994) are quite proteolytic in comparison with calf rennet. Phelan (1977) reported that during curing of Edam,

Tilsiter and butter cheese, protease from *Endothia parasitica* was highly proteolytic. Besides, for production of microbial enzymes from the fermentation media, higher technical know how and facilities are needed (Farmakalids, 1995; Lorenzen *et al.* 1996).

c) Coagulants from other animals

Rennet substitutes from pig, fish, chicken, cattle, goat and sheep have been produced. Porcine, chicken and fish pepsin are highly proteolytic when used as the sole coagulant. But when used as 50:50 blend with calf rennet, they give satisfactory results (Green, 1977). Pepsin from chicken proventricular and fish has been reported to have an excessive proteolytic activity resulting in relatively greater breakdown of cheese proteins. This leads to poor quality of cheese with tendencies to develop off flavours particularly cattiness, bitterness and soft body (Green, 1972, 1977; Phelan, 1977; El-Abbassy *et al.* 1994). On the other hand, the use of porcine rennet is limited due to religious factors among Muslims, Seventhday Adventists and vegetarians who do not use pig products. Therefore, the best alternative to get the rennet substitute for Tanzania would be from adult cattle, goat and sheep.

2.1.2 Pepsin as an appropriate rennet substitute

Calf rennet and bovine rennet have low proteolytic activity and are fairly stable during cheesemaking (Webb *et al.* 1974 and Fox, 1989, Andren *et al.* 1990). Also, McMahon and Brown (1982) reported that proteolytic activity of calf rennet is predominantly due to chymosin (85 - 95%) an excellent milk clotting enzyme which is very rapid and more specific than other rennets. The remainder is bovine pepsin which resembles chymosin but is less specific and might result in undesirable effects in cheese ripening (Andren

and Reedtz, 1990; Visser, 1993). Adult bovine rennet preparations may contain up to 55 - 60% or even more bovine pepsin (Fox and Walley, 1971; Andren and Reedtz 1990). They also reported that there was no significant difference between Cheddar cheese made from bovine rennet and that made from calf rennet. Also, Green (1972) and O'Leary and Fox (1975) reported that cheddar cheese made entirely with bovine pepsin was only slightly inferior to that made with calf rennet. Furthermore, Fox, *et al.* (1989), in their cheesemaking experiments, found that bovine rennet showed greater similarity in its properties to calf rennet than did the porcine pepsin, and suggested that bovine rennet might prove to be the most suitable rennet substitute. Anden and Reedtz (1990) reported that as long as the cheese curd is cut at the proper firmness, there are probably no practical problems in using bovine rennet with varying chymosin/pepsin ratio.

Pepsin is composed of a single polypeptide chain of 321 amino acids residues with a molecular mass of 35,000 Da. Pepsin tertiary structure is stabilised in part by three disulphide bridges and a phosphate linkage which is attached to a hydroxyl group of a seryl residue (Whitaker, 1972; Webb *et al.* 1974; Foltmann, 1987). The enzyme is quite stable in the pH range 2 - 5. Above and below this pH range the enzyme rapidly looses its activity due to denaturation (Whitaker, 1972). The optimum pH is 1.8 - 2 and temperature 43°C. (Davis, 1965; Webb *et al.* 1974). Pepsin has been reported to have a broad specificity for peptide bonds favouring those peptide bonds of adjacent aromatic amino acids (Reed, 1975).

The source of bovine pepsin

The stomach of ruminants is composed of four structurally distinct parts. The first three parts, the rumen, reticulum, and omasum are collectively known as the forestomach and are lined with glandular mucous membrane having a stratified squamous epithelium. The forth part, the abomasum is the glandular portion similar to the simple stomach of other species (Swenson, 1970; Andren *et al.* 1980, 1986). Pepsin, gastricsin and chymosin are produced and secreted by abomasal mucosa as the inactive zymogens pepsinogen, progatricsin and prochymosin respectively (Andren *et al.* 1980, 1986).

The abomasal cavity is divided by a constriction into two regions. In the largest, the fundic region, the gastric mucosa is formed into spiral folds which are longitudinally oriented inside the surrounding muscular wall. The folds are largest in the area nearest the omasum and decrease in size towards the pyloric region, which is the other, narrower, part of the abomasal cavity (Figure 1). In the surface of the gastric mucosa there are a large number of funnel shaped gastric pits. The gastric (oxyntic) glands of the fundic region and the pyloric glands of the pyloric region open into the bottom of these gastric pits (Andren, 1985). There is no sharp border-line between the fundic region and the pyloric region. The different glands intermingle with each other in a limited zone between the regions, that is, chief cells and HCl producing parietal cells decreases in number and mucous cell increase towards the pyloric region (Andren and Björck, 1986).

Andren *et al.* (1981) found that, in concentrate - fed calves and older cattle, almost all mucous neck cells and chief cells situated in the upper part of the base, produce pepsinogen and prochymosin. The number of prochymosin -immunoreactive chief cells

found to be low in concentrate fed calves, correspond to the lower content of chymosin. Although the abomasal mucosa of the cow contained only little chymosin, a relatively large number of chief cells in the upper base contained prochymosin. In older cattle, the chief cells in the lower base seem to have lost the ability to produce prochymosin. This lack of prochymosin in the lower base can not be explained by a missing stimulus for prochymosin production.

The occurrence of prochymosin containing chief cells in the upper base of the abomasa does not correspond with the fact that chymosin has no apparent digestive function in animals not fed with milk. Kataoka (1970), came up with a plausible explanation that, the chief cells situated in the lower base may be further differentiated compared to the cells in the upper base and then have changed their pattern of zymogen production. In the pyloric of the young milk fed calves, prochymosin and pepsinogen was found to a greater extent in the lower parts, while only pepsinogen was found in the same area of the abomasa of older cattle (Andren, 1985). To what extent concentration of pepsin is in the fundic and pyloric region of the adult cattle was a subject for further investigations (Andren *et al.*1980).

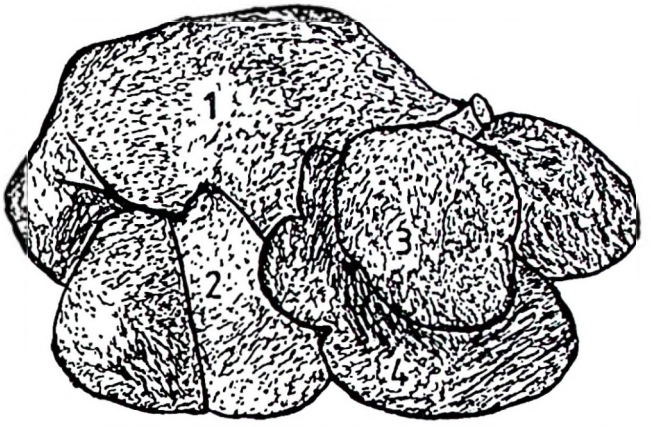
KEY

1 Dorsal sac rumen

2 Ventral sac rumen

3 Omasum

4 Abomasum



F - Fundic

P - Pyloric

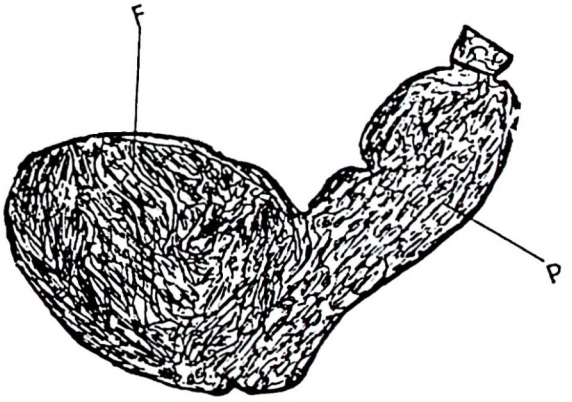


Fig. 1: Ruminant digestive system and location of abomasum: fundic and pyloric regions (Getty, 1975).

2.1.3 Chymosin and pepsin production in the cattle abomasa

Pepsin is produced in the abomasa of an adult cow. When a calf is born, chymosin is present in the gastric mucosa at 2 - 3 mg/g but its production declines after a week (Andren, *et al.*1981), unless the calf is continuously fed milk (Andren and Björck, 1986). During the 1st week of life pepsin is virtually absent, but rapid increase in its production takes place after about 3 weeks (Webb *et al.* 1974). This continues until the

pepsin replaces chymosin as a principal gastric enzyme of the bovine (McMahon & Brown, 1985; Andren and Björck, 1986). Chymosin and pepsin are chiefly located in the fundic region of the abomasum, while the pyloric region contains very little of these enzymes. This occurs with the anatomical-histological differences in the abomasum, as the pyloric region mostly contains mucin producing cells, while the fundic region contains proportionally more enzyme producing cells. Andren *et al.* (1981) also observed that the middle part of the abomasum of concentrate-fed calves and heifers contain more chymosin than the other parts of the fundic region.

From the analysis by Andren *et al.* (1980, 1982) it was found that in concentrate - fed calves (about 6 months old) the amount of chymosin decreased drastically although they still contain 2-5 mg /g mucosa. Chymosin decrease further in heifers (12 - 24 months old) and constitute only 1 to 2 percent of the total amount of proteolytic enzymes. In the abomasa from cows (older than 24 months) only traces of chymosin were found in about half of the material analysed. Generally, the variation of enzyme content in the abomasa within the different groups of cattle may to some extent depend on the different times between the last feeding and slaughter (Hagyard and Davey, 1974) also on individual ability to produce enzymes.

2.1.4 Conversion of pepsinogen to pepsin

Pepsinogen is produced by chief cells of the stomach wall and is secreted into the stomach in the presence of HCl where it is converted to active enzyme pepsin. The conversion of pepsinogen to pepsin is catalysed by pepsin and results in the release of a number of peptide fragments from the N-terminal end of pepsinogen (Whitaker, 1972). Fig. 2 shows points which are hydrolysed during the formation of pepsin and the release

of other peptides of low molecular weight (Whitaker, 1972; Webb *et al.* 1974). Nielsen and Foltmann (1993) quoting James and Sieleck (1986) suggested two possible paths of conformational change during activation, one in which an exchange of N-terminal residues occurs after proteolytic cleavage and one in which an exchange of N-terminal in the β sheet occurs before proteolysis. It is likely that only one of the bonds is necessary to release the active enzyme (Whitaker, 1972).

These peptides are derived from 44 amino acids at the amino terminal portion. One of these peptides with 29 amino acid residues acts as pepsin inhibitor above pH 5 by forming a pepsin inhibitor complex (MM 5000) (Reed, 1975; Fox, 1987). The inhibitor is bound to pepsin between pH 5 - 7 and inhibits both the clotting activity and protein digestion power of the enzyme. At pH 2 - 5 the inhibitor dissociates from the enzyme and digestion power of the enzyme is restored (Swenson, 1970; Whitaker, 1972; Webb *et al.* 1974).

A - Peptide

B - Pepsin inhibitor

C - Pepsin

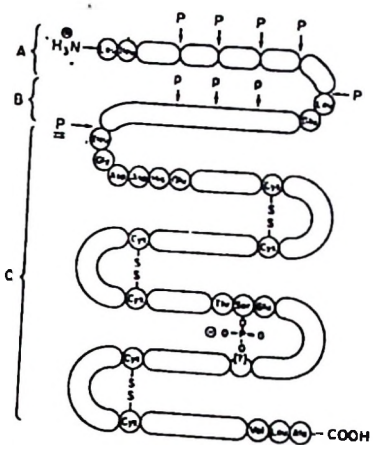


Figure 2: Schematic representation of the structure of pepsinogen and its conversion to pepsin. The major points of hydrolysis are marked with P and result in release of several peptides (A), pepsin inhibitor (B) and pepsin (C). Hydrolysis of the bond P is essential for activation (Whitaker, 1972).

During the conversion of pepsinogen to pepsin, the molecular mass decreases from 42,000 to 35,000 Da and the isoelectric point changes from 3.7 to less than 1. This is due to the loss of 9 of the 10 lysyl residues, 3 of the 3 histidyl residues and 2 arginyl residues during activation (Webb *et al.* 1974; Whitaker, 1972; Fox, 1987). Conversion of pepsinogen to pepsin is best in the pH range of 1 - 2 (Whitaker, 1972). There is no activity at pH 8 (Whitaker, 1972).

Pepsin and other gastric proteases are synthesised and secreted as zymogens which are converted into active enzymes in the acidic gastric juice. The first systematic experiments on the activation of pepsinogen were carried out by Herriott in 1939 and after subsequent experiments, pH 2 was found to be appropriate and was taken as measure for conversion of pepsinogen to pepsin (Nielsen and Foltmann, 1993) while Foltmann, (1981) obtained maximum MCA at pH 3. Activation process is initiated by a pH-dependence change into active conformation without cleavage of peptide bonds then the reaction proceeds by limited proteolysis that finally remove 44 amino acid residues (Harboe *et al.*1974) from the N-terminal end of the peptide chain which mainly cleaves at Leu16p and Ile17p. Several studies gave different cleavage sites and reported that the process was complex (Foltmann, 1960, 1981; Nielsen and Foltmann, 1993). Pepsin inhibiting substances formed during activation have been shown to correspond to NH_2-terminal of 16 and 17 amino acids (Harboe *et al.*1974). Moreover Foltmann, (1960) found out that activation was accompanied by hydrolysis of peptide bond near the NH_2-terminal end of the molecule and the enzyme activity increased with the time after activation. Rajaopalan *et al.* (1996) described this as the result of the formation of active species when activation pH was low. James and Sielecki (1986) suggested two possible pathway of conformational change during the activation: one in which an exchange of N-terminal residues occurs after proteolytic cleavage and the other an exchange of N-temini in the b-sheet occurs before proteolysis. Results by Nielsen and Foltmann (1993) revealed the non-covalent intermidiates between prosegments peptides and pepsin. The complexes Leu1p-Leu44p/pepsin and Leu1p-Leu16p/ pepsin were isolated (the prosegment residues are characterised by suffix p; numbering of residues starts again from the N-terminus of pepsin). The acid

producing pariental cells are located mainly above the chief cells. This means that the zymogen of the gastric protease is secreted directly into a highly acidic liquid and under the physiological conditions the initial limited proteolysis of the activation process presumably occur already in the lumen (Andren *et al.* 1981, 1986).

Summary of conversion of pepsinogen to pepsin.

1. Pepsinogen --------^{pepsin}-------> Pepsin-inhibitor complex + 5 peptides

11 Pepsin-inhibitor complex ----------> pepsin + inhibitor

<----------

111 Inhibitor -----^{pepsin}----------> Pepsin + inhibitor

(Dixson and Webb, 1967)

2.1.5 Extraction of crude bovine pepsin

a) Breakage of mucosal cells

As soon as the fresh abomasa are obtained, simple breakage of cells is normally done fast by homogenisation in warring blender (Green, 1972; Whitaker, 1972). Dried abomasa are normally minced or ground or cut into very small pieces and then mixed with extraction solution or buffer of high ionic strength (Davide *et al.*1982; Harboe *et al.* 1974).

b) Role of high ionic strength of NaCl

Usually a ration of 1:2 or 1:3 mucosa to buffer is used. Buffer is needed to protect the enzyme from large quantities of acids released from the vacuole from the raptured cells. In most cases NaCl are incorporated to increase the ionic strength, which is important

for increasing the yield and activity of the enzyme (Whitaker, 1972; Surender and Dutta, 1981; Visitera and Isyrenova, 1985). It is also required to release the enzyme from its attachment (Scrimgeour, 1977). Surender and Dutta (1981) reported that extraction of pepsin using NaCl required 10 hr and 8 hr with $CaCl_2$ whose activities were 11.64 RU/ml and 7.6 RU/ml respectively.

In Visatera and Iysrenova (1985) experiment on yield and activity testing of pepsin enzyme from sheep abomasa, it was shown that the yield increased by increasing the edible NaCl from 5 to 10% to the extraction solution. NaCl is commonly used, probably due to the fact that it is one of the ingridient in normal diets of the human being. $CaCl_2$ also has been used. This may be due to the fact that Ca^{++} ions are essential to the formation of a coagulum of the paracaseins which have been converted from casein by the enzyme, adsorbs Ca^{++} which in turn link the paracasein to form a network in which whey is enclosed. Results obtained by Anis *et al.* (1983) showed that there was direct relationship between the amount of NaCl added from the lower amount to about 10% and the clotting time of milk. Clotting time increases progressively with the amount of NaCl added. Such observation was true in all types of rennet in both kinds of milk with different levels of NaCl. This finding is in agreement with the known theory of the partial salting out of rennet enzymes as well as the cation exchange taking place between sodium and calcium ions in milk.

c) Effect of extraction time on milk clotting activity

Milk clotting activity (MCA) expressed as rennet unit per ml (RU/ml) or Total Rennet Unit (RU) and proteolytic activity increases gradually up to 8 days then decreases on

day 10 of extraction period of adult bovine rennet (Hewedy *et al.* 1992). Such an increase is expected because the release of protein containing enzymes from tissue could be time dependent. A similar finding was obtained by Davide (1983). The activity of the enzymes increased from the first day of extraction to the fifth day. A good result of protein content to milk clotting activity (MCA) and proteolytic activity was obtained by centrifuging at 5000 g/15 minutes (Hewedy *et al.* 1992).

d) Effect of method of drying abomasa on MCA

A number of studies have been conducted towards finding how to increase and improve abomasa extract in terms of yield and its activity on milk clotting during cheesemaking. It was found that extracts from dried abomasa was about 2% higher in MCA than in fresh abomasa mucosa (Lyatuu, 1991). Drying of the unextracted material is essential for yield increase (Davide, 1983). It is presumed from this that drying has the effect of modifying the enzyme converting it to a more active form. Hagyard and Davey (1974) investigated that drying of abomasa reduces the viscous nature of the extraction process, increases the yield of pro-chymosin and preserves the enzyme. The result was similar to that obtained by Hewedy *et al.* (1992) that dried cuts showed higher MCA than fresh minced mucosa.

e) Effect of extraction temperature on MCA

The process of converting pepsinogen to pepsin is an enzymatically controlled reaction and thus can be influenced by temperature. Wigenge (1989) reported that crude pepsin extracted at room temperature, 20 - 24°C gave higher values of enzymatic activity than those extracted at ice cold temperature. Thus conversion of pepsinogen to pepsin takes

place under physiological condition, 37°C (Fox, 1987; Surender and Dutta, 1981). Heating the crude extract below the optimum temperature for the enzyme is desirable to denature and precipitate unwanted protein.

f) Effect of extraction pH on MCA

For in-vitro extraction of crude bovine, dilute HCl of pH 6 - 7 is used as extraction solution. Conversion of pepsinogen to pepsin is achieved by lowering the pH to 2. Surender and Dutta (1981) used 0.04 M HCl while Chow and Kassel (1969) used 0.3 M HCl. Use of HCl could be due to the fact that in the animal, the natural conversion of pepsinogen to pepsin is facilitated by natural HCl present. Davide (1983) and Peralta *et al.* (1985) extracted the enzyme using Vinegar of pH 2.6 from Carabao cattle and goat abomasa. Herrian *et al.* (1983) found that using dilute HCl at pH 1.4 gave best results.

g) Clearing or pre-purification methods of crude extracts

Whitaker (1972) Green (1972) reported clarification using centrifugation at 1500 g for 30 min. Whitaker (1972) used filtering through Whatman paper No 40 to remove precipitated materials. Surender and Dutta (1981) used $NaHPO_4$ to raise the pH to 5.3 - 6.3 to remove objectionable odour, mucin and other insoluble materials and get a clear yellow solution and used the centrifuge to separate the two and sedimentation. Rye (1969) used NaCl at 25% concentration to precipitate the material while Whitaker (1972) reported that heating the crude extract to about 30°C denatures some of the mucosa material precipitating them leaving a clear solution. Also NaOH, $NaHPO_4.2H_2O$ and $NaHCO_3$ has been used (Rye, 1969)

2.1.6 Characterization of crude pepsin extract

a) Determination of milk clotting activity

The International Dairy Federation Standard 157:1992 specifies a method for determination of the total milk clotting activity at pH 6.5 of chymosin and bovine pepsin in bovine rennets.

Its importance is that it characterises the coagulants relative to an internationally recognised standard control rennet with known composition and milk clotting activity (Andren, 1992).

b) Hydrolysis of caseins by coagulants

The enzymatic step of the milk clotting process can be followed by the release of TCA-soluble N from milk, whole casein or κ-casein. The sensitivity of this method can be increased by adjusting the pH value to the optimum for the enzyme to be assayed and choosing the appropriate protein substrate. The problem may arise that different coagulants can hydrolyse unidentified bonds in the substrates which may vary with different enzymes (Green, 1977; Visser, 1993; Ustunol-Z and Zeckzer, 1996). Rate of hydrolysis of whole casein and production of fractions and properties of the hydrolytic products in comparison with the rate of release of 12% TCA soluble N after incubation of milk with coagulant at pH 6.5- 6.7 and the number of peptide bonds hydrolysed suggests which coagulant is more proteolytic than the other. Most of the research has shown that all other coagulants are slightly more proteolytic than rennet (Andren and Reedtz, 1990; McSweeney *et al.* 1993; Ortz de Apodaca *et al.* 1994).

c) Casein-agar diffusion diameter

The casein agar diffusion technique was developed by Cheeseman in 1963 to measure both qualitatively and quantitatively proteolysis of extracts. Diffusion of coagulant from the central well through a suspension of Ca-caseinate in agar causes 2 zones to develop. The outer white zone represents the precipitation of Ca-paracaseinate and the inner zone is the region of general hydrolysis. Lawrence and Sanderson (1969) and Green (1977) reported that the outer zone was associated with breakdown of κ-casein to para-k-casein and the inner zone show that extensive degradation of the major caseins has occurred. Solutions with low proteinaese activity show little or no ability to further degrade the white zone around the well. Enzymes producing larger inner transparent zones relative to outer white zone are unlikely to be suitable coagulants for they would degrade the clot too readily.

2.2 Enzyme purification

Serious progress in purification of enzymes dates from about 1922 by Northrop. The first crystalline enzyme urease (3.5.1.5) was obtained by Sumner in 1926, and by 1940 about twenty highly purified enzymes had been obtained and the process has continued since at an ever increasing rate (Fox, 1987). At the present time, the total number of purified enzymes is many hundreds (Fox, 1987; Foltmann, 1992). Bovine pepsin was first isolated by Northrop in 1933 (Meitner and Kassell, 1971).

Several techniques are employed in the purification of proteins ranging from simple to very sophisticated methods and from those that can be used in a small scale to those for large scale purification (Andren, 1992; Price and Steevens, 1991; Foltmann, 1992; Verissimo *et al.* 1996 and Asakura *et al.* 1997).

Kuchoo and Fox (1982) reported that it was frequently impossible to separate interfering activities from enzyme preparations by single stage processes. However, a series of chromatographic processes and portion processes are available for purification (Asakura *et al.* 1997). Enzyme extracts contain numerous other substances of both large and small molecular weight. Small molecules can be removed by dialysis or ultrafiltration leaving large molecules, predominantly proteins, though some polysaccharides may be present.

The main part of purification consists of a series of fine fractionations by which the enzyme protein is separated from other proteins present. Fractionation must always be monitored and controlled by activity testing (Whitaker, 1972; Fox and Whitaker 1977; Verissimo *et al.* 1996). The methods can be grouped into the following categories.

2.2.1 Properties and methods used in separation and purification of enzymes

Table 2: A summary of properties and methods used in separation and purification of enzymes (Price and Stevens, 1991):

Property	Method	Scale of method	
		Large	Small
Solubility	Change in pH	1	-
	Change in ionic strength	1	1
	Size or mass		
	Centrifugation	1	1
	Gel filtration	-	1
	Dialysis/ultrafiltration	-	1
Charge	Ion exchange chromatography	1	1
	Electrophoresis	-	1
	Hydrophobic chromatography	-	1
Specific structural features	Affinity chromatography	1	1
	Dye ligand chromatography	1	1

a) <u>Solubility</u>

Protein solubility varies and can be used for separation purposes as determined by 3 main factors:

- Charge density and charge distribution

- Degree of hydration
- Presence of non protein components of a molecule such as phosphate, CHO and lipids.

These methods may be combined with other methods like hydrophobic or ion exchange chromatography to separate components by size or mass accordingly (Chow and Kassel, 1969).

Decrease in dielectric constants

Charge density of a protein is determined largely by the number of acidic and basic amino acid residues. Because of the hydrophilic nature of these groups, they are generally oriented on the outside of a protein molecule so as to be exposed to the aqueous phase. Charge density of a protein can be affected by changing the pH (Foltmann, 1992).

At low pH, all the prototypic groups will be protonated and the protein will have a net positive charge. As the pH increases more and more of the carboxyl groups on the proteins are neutralised until there are equal numbers of + and - with a total charge of zero called isoelectric point (Whitaker, 1972; Fox, 1977). Most proteins are least soluble at their isoelectric point. Solubility increases as the pH is decreased below and increased above the isoelectric point. Attractive forces between two molecules are directly proportional to the point of the positive Z^+ and negative Z^- charge on the two molecules which is at a maximum or at an isoelectric point and inversely proportional to the dielectric constant of the medium. All other factors being constant, lowering the dielectric constant of the solution decreases, thus

attractive force such as acetone or alcohol are used to precipitate proteins (Nevaldine and Kassel, 1971); Whitaker, 1972; Foltmann, 1992).

Change in ionic strength

This is the relationship between solubility of a protein and NaCl concentration expressed as ionic strength. Solubility of a protein is also determined by the extent of hydration of the molecule. Although the degree of hydration may be changed to some extent by varying the pH, the usual way of decreasing the degree of hydration is to add compound which disrupt the water of hydration around the protein such as ammonium sulphate, sodium chloride and sodium sulphate. The former is mostly used because of its high solubility in water. At low ionic strength there is a salting-in effect but as the ionic strength is increased a range is reached where the protein begins to be less soluble (Garnot *et al.*1972; Fox, 1993; Abdel-Malak *et al.*1996).

Precipitation

This is the method of separation by which total protein is separated into a series of fractions by gradually increasing the NaCl concentration, or the amount of adsorbent added or the concentration of organic solvent or the acidity in such a way that the greater part of the proteins is precipitated. This method is usually used to remove unwanted proteins (Castle and Wheelock, 1971; Foltmann, 1992).

Fractional precipitation by change of pH

Although not one of the important fractionation methods, it is often very advantageous with extracts of animal tissues. The pH is adjusted to pH 5, allowed to

stand for few minutes and centrifuged as the first step before the main purification begins. The removing of much nucleoprotein and particulate material often converts a turbid extract into a perfectly clear solution. Occasionally an enzyme may be precipitated in this way (Nevaldine and Kassel, 1971; Abdel-Malak *et al.*1996).

b) Size or mass

Solid - liquid separation is a central basic operation in isolation of enzymes. This step is necessary in separation of cells from the culture broth. It is also used in the clarification of the crude extract after disintegration of the cells and elimination of cell fragments of precipitates after a precipitation operation. Sometimes solid-liquid separation is used in removing added adsorbents from protein-containing solutions. Two procedures are available for solid-liquid separation: centrifugation and filtration.

Centrifugation

After the enzyme has been extracted from the tissues, the cellular debris are removed by high speed centrifugation at 10,000 - 20,000 g for 20-30 min usually in a refrigerated centrifuge. After centrifugation the supernatant liquid is analyzed for both remaining protein and activity. There are many types of centrifuges for different separation levels (Whitaker, 1972 and Prave *et al.* 1987).

Filtration procedures

Dialysis

Dialysis is for elimination of impurities with low molecular weights from protein solutions. The semipermeable membranes used are generally cellulose acetate tubes

(Whitaker, 1972) of molecular mass cut-off 10,000 kDa. The transport of the molecules takes place here exclusively by diffusion and therefore conventional dialysis is very time consuming and requires large amounts of water or buffer solution. This method is very much used in several steps during purification of enzymes. Dialysis has the advantage of an uncomplicated mode of operation and very inexpensive apparatus. To accelerate the process more favourable ratios of surface to volume and adequate mixing of retentate and diffusate must be sought. The boundaries between dialysis and diafiltration are the fluids used (Prave *et al.* 1987). Diafiltration is the name given to an ultrafiltration process when the issuing permeate is continuously replaced by a supply of water or a buffer of known composition.

Ultrafiltration

Ultrafiltration is used for the isolation of intracellular enzymes as well as for concentration to reduce the volume of the process solution. On the industrial scale ultrafiltration has become the method of choice as a simple and efficient process step for concentration.

Electrical Charge

Electrophoresis

Electrophoresis is used more to test the homogeneity of protein preparations than as a method of preparation on any considerable scale. It is very useful for following the progress of the purification and very valuable for obtaining very pure small samples of the enzyme so that its specific activity can be measured, and it is used to

determine the purity of various fractions. Electrophoresis on paper has been used for enzyme separation. Foltmann (1966) used paper electrophoresis to purify prochymosin separated into two almost equally large fractions. Zone electrophoresis in starch gels has been successfully used for small scale isolation of enzyme in the final stages of purification (Fox, 1987).

Polyaccrylamide gel electrophoresis (PAGE) is commonly used today. Sodium dodecyl sulphate - PAGE (SDS-PAGE) has been commonly used to determine molecular mass of enzyme subunits and commercial mixtures of molecular mass markers are available.

Affinity chromatography

This principle covers processes which make use of biospecific binding between enzymes and their ligands as the basis for separation (Prave *et al.*1987). It is increasingly used in enzyme purification because it is more rapid giving high yield, activity and can lead to pure enzyme with a minimum of purification step, (Nevaldine and Kassel, 1971; Foltmann, 1987; Asakura *et al.* 1997). The enzyme is passed through a column prepared on an insoluble matrix to which is attached a group of ligand for which the enzyme has specificity. The enzyme will be retarded because of its interaction with the attached ligand, e.g. substrate - like compound, while the other proteins will pass directly through the column. The desired enzyme can be eluted from the column later by changing the ionic strength, pH or dielectric constant of the eluting solvent. Abdel-Malak *et al.* (1996) isolated chymosin from buffalo abomasa by affinity chromatography. The buffalo enzyme resembled calf chymosin (Chow and Kassel, 1969; Nevaldine and Kassel, 1971).

d) Ion Exchange chromatography

Principle of ion exchange chromatography

The principle feature of ion exchange chromatography is the attraction between opposite charges of proteins and a solid matrix. Adsorbed proteins are gradually eluted according to the charge with NaCl gradient or pH gradient (Whitaker, 1972; Garnot *et al.* 1972; Pharmacia, 1993).

The first stage is equilibration in which the ion exchanger is brought to a starting state, in terms of pH and ionic strength, which allows the binding of the desired solute molecules. The exchanger groups are associated at the time with exchangeable counter - ions (usually simple anions or cations, such as chloride or sodium) (Chow and Kassell, 1969; Fox and Whitaker, 1977; Nielsen and Foltmann, 1993).

The second stage is sample application and adsorption in which solute molecules carrying the appropriate charge displace counter ions and bind reversibly to the carrier. Unbound substances can be washed out from the exchanger bed using starting buffer (Garnot *et al.* 1972).

In the third stage, substances are removed from the column by changing to elution conditions unfavourable for ionic bonding of the solute molecules. This normally involves increasing the ionic strength of the eluting buffer or changing its pH. The desorption is often achieved by the introduction of an increasing NaCl concentration gradient and solute molecules are released from the column in the order of their strengths of binding, the most weakly bound substances being eluted first (Garnot *et al.* 1972; Whitaker, 1972).

The fourth and fifth stages are the removal from the column of substances not eluted under the previous experimental condition and re-equilibration at the starting conditions for the next purification (Garnot *et al.* 1972; Whitaker, 1972).

In ion exchange chromatography one can choose to bind the substances of interest and allow the contaminants to pass or to bind the contaminants and allow the substances of interest to pass through. Generally the first method is more useful since it allows a greater degree of fractionation and concentrates the substances of interest (Whitaker, 1972). In addition to the ion exchange effect, other types of binding may occur. These effects are small and are mainly due to van der Waals forces and non-polar interactions (Whitaker, 1972).

Ion exchanger matrix

An ion exchanger consists of an insoluble porous matrix to which charged groups have been covalently bound. The charged groups are associated with mobile counter ions. These counter-ions can be reversibly exchanged with other ions of the same charge without altering the matrix. It is possible to have both positively and negatively charged exchangers. Positively charged exchangers have negatively charged counter ions. Negatively charged exchangers have positively charged counter-ions and are termed anions and cations, respectively.

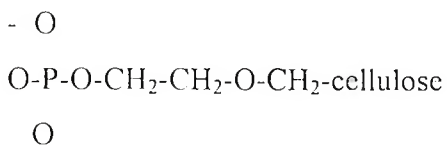
Example of anion exchanger (Whitaker, 1972):

Diethylaminoethyl-cellulose (DEAE)

$$(CH_3\text{-}CH_2)_2\text{-}N\overset{H}{\overset{|}{}}\text{-}CH_2\text{-}CH^3\text{-}O\text{-Cellulose}$$
$$\overset{+}{}$$

Example of cation exchanger:

Phosphoethyl-cellulose (PE)



The matrix may be based on inorganic compounds, synthetic resins, polysaccharides etc. The characteristics of the matrix determine its chromatographic properties such as, capacity and recovery as well as physical properties such as its mechanical strength and flow properties. The nature of the matrix will also affect its behaviour towards biological substances and the maintenance of biological activity (Pharmacia 1993).

The first ion exchangers were synthetic resins designed for applications such as demineralisation, water treatment, and recovery of ions from wastes. Such ion exchangers consists of hydrophobic polymer matrices highly substituted with ionic groups, and have very high capacities for small ions. Due to their low permeability these matrixes have low capacities. Charge density gives very strong binding and the hydrophobic matrix tends to denature labile biological materials. Thus despite their excellent flow properties and capacities for small ions, these types of ion exchangers are unsuitable for use with biological samples (Whitaker, 1972).

The first ion exchangers designed for use with biological substances were the cellulose ion exchangers developed by Peterson and Sober in 1956. Because of the hydrophilic nature of the cellulose, these exchangers had little tendency to denature proteins. Unfortunately many cellulose ion exchangers have low capacities

(otherwise the cellulose became soluble in water) and poor flow properties due to their irregular shape (Pharmacia, 1993).

Sepharose based ion exchangers

Fox and Whitaker (1977); Asakura *et al.* (1997) reported that Sepharose polylysine gave excellent recovery of enzyme with a high purification factor. Ion exchangers based on dextran (Sephadex), followed by those based on agarose (Sepharose CL-6B) and cross-linked cellulose (DEAE Sephacel) were the first ion exchange matrices to combine the correct spherical form with high porosity, leading to improved flow properties and high capacities for macromolecules. Subsequently, developments in gel technology have enabled this macroporosity to be extended to the highly cross linked agarose-based media such as Sepharose Fast Flow and Sepharose High Performance, and the synthetic polymer matrix, MonoBeads. These modern media offer the possibility of fast, high capacity, high resolution ion exchange chromatography at both analytical and preparation scale (Garnot *et al.* 1972; Fox and Whitaker, 1977; Verissimo *et al.* 1996).

Partial structure of Sepharose CL-6B ion exchanger

$$\text{DEAE (Sepharose CL-6B-O-CH}_2\text{CH}_2\overset{\overset{C_2H_5}{|}}{\underset{\underset{C_2H_5}{|}}{N}}\text{-H}\quad Cl^-$$

iv) MonoBeads

This ion exchanger is designed for fast, high resolution separations of proteins, peptides, polynucleotides with fast flow liquid chromatography (FPLC) systems. They are based on a beaded hydrophilic resin with the narrowest particle size distribution available for chromatographic matrixes (+/-0.5mm) (Pharmacia, 1993).

Mono Q is a strong anion exchanger based on MonoBeads which bind negatively charged biomolecules through quaternaryramine groups that remain equally charged over the entire useful pH range of 2-12. Mono Q is especially useful in isolation of monoclonal antibodies. Nielsen and Foltmann, (1993) purified porcine gastric mucosa on Mono Q with high recovery. Thousands of enzymes have been purified on Mono Q (Pharmacia, 1993).

Hydrophobic interaction chromatography (HIC)

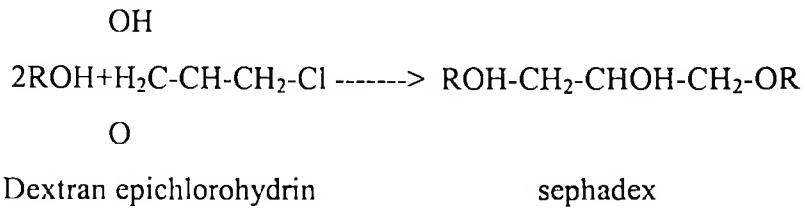
HIC is a versatile method for purification and separation of biomolecules based on differences in their surface hydrophobicity. Pre-packed HiLoad columns of Phenyl Sepharose High Performance is based on highly cross-linked agarose beads derivatized with phenyl groups via uncharged, chemically stable ether linkages (Pharmacia, 1993).

Gel Filtration chromatography

Gel filtration is a simple and reliable chromatographic method for separating molecules according to size (Whitaker, 1972; Fox and Whitaker, 1977). Its versatility makes it generally applicable to the purification of all classes of biological substances, including macromolecules not readily fractionated by other techniques. Good separations and high activity can be obtained. Molecules larger than the largest pores in the swollen gel bed (those above the exclusion limit) cannot enter the gel bead and are eluted first. Smaller molecules which enter the gel beads to varying extents, depending on their size and shape, are slowed on their passage through the bed. Molecules are eluted in a predictable way in order of decreasing

molecular size, making gel filtration a useful non-destructive method for estimating molecular size (Rajagopalan *et al.* 1996; Whitaker, 1972; Verisimo *et al.*1996).

Partial structure of Sephadex gel:



Pharmacia (1993) and Whitaker (1972) gave details of the different selections of Sephadex for gel filtration, molecular weight fractionation range for proteins to be applied on ion exchange chromatography (Rothe *et al.* 1976).

2.2.2 Order of purification by ion exchanger

There are different steps which can be followed during purification of enzymes. However, there is no hard and constant rule that governs the sequence of selected steps, (Fox, 1987; Foltmann, 1992). The establishment of the reproducible and efficient purification can be challenging in itself. Whichever method it might involve, a simple breakage of the cells to release the enzyme must be done first (Whitaker, 1972; Dixon and Webb, 1967; Foltmann, 1992).

Whitaker (1972) suggested the order of purification as follows:

1. Crude extract be centrifuged to remove suspended materials.
2. Adjust the pH by addition of protamine sulphate or streptomycin to remove nucleic acid.
3. Concentrate by partial freezing or use of UF or fractional precipitation.

4. Dissolve material in small water or buffer volume.
5. Apply gel filtration or dialysis to remove NaCls.
6. Pool fractions with highest activity from gel filtration, concentrate by UF or precipitation.
7. Dialyse overnight with buffer to be used for the next chromatography.
8. Elute on ion exchange with linear gradient.
9. Pool, concentrate, dialyse the most active fractions from chromatography.
10. Chromatography on ion exchanger.
11. Fractionate, pool concentrate and test for homogeneity or if not pure continue purification.
12. Pure enzyme to be stored at 0°C or -20°C while storage experiments continue to check loss of activity.

2.2.3 Determination of conditions for adsorption and elution of enzymes on ion exchanger.

- Preliminary experiments which defines good conditions for elution of proteins must be done first (Whitaker, 1972).
- For separation of chymosin and bovine pepsin, preparation of the column has been recommended by IDF Standard 110A: 1987. First it is washed with 0.25 M NaCl then 0.25 M NaOH then two water washings between them with 0.25 M HCl then water until chloride free and finally equilibration against starting buffer for chromatography (Price and Stevens, 1991).
- The NaCl concentration changes slowly in the region where the enzyme peak is eluted so that it is well separated from other protein peaks (Whitaker, 1972).

- The dimensions of the column also have been reported to determine the capacity and resolution of the purification on ion exchange chromatography. The more the protein to be added to the column the larger should be the volume of the column. Two centimeter diameter column can handle 1 g protein quite well.
- Solubility of proteins is determined by the extent of hydration. Phosphate and carbohydrates groups increase while lipids decrease the degree of hydration.
- Increasing the hydration disrupts the water around the protein which is usually done by addition of ammonium sulphate, sodium chloride, sodium sulphate, magnesium chloride. Ammonium sulphate is by far the most used because of its high solubility in water Whitaker (1972).

During the purification of enzymes, there are some basic consideration which must be followed to avoid inactivation by denaturation of enzymes. Some of these are: maintenance of cold conditions to prevent autolysis or microbial growth and use of buffers to protect the enzyme from fluctuations in pH. Anti-microbial agents can be incorporated into the extraction solution but should have no negative effect to the enzyme solution (Dixon and Webb, 1974; Whitaker, 1977; Andren, 1992; Price and Stevens, (1991). The step which cause denaturation of the enzyme should be eliminated and appropriate steps taken (Whitaker, 1972).

2.2.4 Isolation and purification of chymosin and pepsin

Foltmann (1966) and Abdel-Malak *et al.* (1996) reported that purification of prochymosin by ion exchange chromatography on a column of DEAE cellulose proved to be markedly more effective than other methods. Nevaldine and Kassel

(1971) and Garnot *et al.* (1972) reported that the DEAE cellulose method was quantitative, quick, simple and reproducible for chymosin and pepsin provided that the same batch of low heat skim milk powder is used in milk clotting activity tests. Ion exchange chromatography is capable of separating macromolecules with minor differences in properties e.g. two proteins differing by only one charged amino acid (Garnot *et al.* 1972; Verissimo *et al.*1996; Asakura *et al.*1997).

DEAE cellulose chromatography has been selected by the International Dairy Federation to be used as a standard method for determination of chymosin and pepsin content of rennet preparations (IDF 110A: 1987; Andren, 1992). Only ion exchange chromatography methods which are used to purify pepsin or rennet will be reviewed because they were used in this study.

2.2.5 Quantification of purification steps and characterization of the purified extract

The success of every step of purification procedure must be ascertained by determining enzyme activity and protein concentration and comparing the two (Whitaker, 1972; Price and Stevens, 1991; Foltmann, 1992).

a) Measurement of enzyme activity in chromatographic fractions

The milk clotting activity of an enzyme is usually determined by measuring the length of time required to coagulate a sample of milk under defined conditions of temperature and pH (Ranken, 1984; Fox, 1989). Normally low heat spray dried skim milk reconstituted with 0.1 M $CaCl_2$ solution to pH 6.4, is used as substrate and

clotting is done at 30°C using 0.5 ml enzyme solution to 25 ml milk (IDF Standard 157: 1992).

Purification is continued until homogenous enzyme of highest specific activity - milk clotting activity per unit weight of protein is obtained (Fox, 1987; Foltmann, 1987; Price and Stevens, 1991). Formagraph has recently been used in determining the curd firming rate of purified proteinases (Andren and Reedtz, 1990).

b) Electrophoresis

Two common determinations of purity are polyacrylamide gel electrophoresis and isoelectric focusing. In these techniques components are separated due to their varying behaviour under the influence of an electric field. If proteins migrate as a single band at two or more pH values and over a range of gel concentrations sufficient to cause separation on the basis of size as well as charge, the protein is considered to be pure (Dixon and Webb, 1967; Garnot *et al.* 1972; Fox, 1987; Foltmann, 1987).

c) Protein concentration

In each step of purification, protein concentration must be determined and expressed as milligrams protein or nitrogen per ml. When units of enzyme per ml are divided by mg protein per ml, the result is specific activity (Whitaker, 1972). It is easy to follow up the degree of purification by comparing the specific activity at that step with the specific activity of the original extract. The ratio of the two gives fold purification. Fold purification is not an index of purity of an enzyme, it only reflects the increase in specific activity (Whitaker, 1972; Foltmann, 1987).

The method used in protein determination at each step of purification should be relatively precise, rapid and not use too much sample for assay (Whitaker, 1972). Protein concentration measurement by absorbancy or optical density at 280 nm is normally used. Absorbency at 278 nm of 1.00 ml containing 100 clotting unit per ml corresponds to 1.43 clotting units per mg chymosin (Foltmann, 1987).

d) Chromatographic behaviour

Important information about purity of an enzyme can be obtained from the symmetric and specific activity of the protein elution peak (O'Leary and Fox, 1975). In a pure preparation, the protein peak is quite symmetrical and the specific activity is constant over the whole peak which is not true of the impure preparation where the impurities are coming off the column slightly later than the enzyme. Special chromatographic procedures on columns containing covalently-bound enzyme inhibitors have permitted a determination of purity of the enzyme preparation on the basis of their active site properties (Whitaker, 1972).

e) Molecular mass determinations

Molecular mass of chymosin and pepsin have been reported to be from 30,400 to 40,000 Da respectively (Whitaker, 1972; Foltmann, 1987, Abdel-Malak *et al.*1996). After determination of the primary structure of calf chymosin, pig pepsin and bovine pepsin, their molecular weights were found to be 35,600, 34,600 and 35,000 Da respectively (Deyl, 1979 and Szecsi, 1992).

f) Phosphate content

Pure bovine pepsin has been reported to contain up to 3 phosphate groups (Martin and Core, 1981 and Szecsi, 1992) but their location in the structure has not been determined (Foltmann, 1987; Szecsi, 1992). The only differences detected among them were the organic phosphate content ranging from 0.3 to 3 mol/ mol and therefore at this content accounted for the 3 chromatographic peaks.

g) Amino acid sequence

Foltmann (1987) reported that only partial amino acid sequences are known. Absence of lysine in the purified sample is a good test of homogeneity (Nevaldine and Kassell, 1971). Fox and Whitaker, (1977) reported that isoleucine is well established as a predominant N-terminal residue while alanine is C-terminal residue for pepsin (McMahon and Brown, 1985; Andren and Reedtz, 1990; Abdel-Malak *et al.*1996)

2.3 Common cheeses in Tanzania

Two types of cheese are commonly produced in Tanzania. These are Pasta filata and Alpine and Gouda . Pasta filata type of cheese is made by combining casein bound with a desired level of calcium, soluble protein and other ingredients to form a mixture which is then elasticised to form cheese. It is particularly suitable for use in pizza manufacture (Yee, 1990; Kindstedt, 1996). Pasta cheese originates from Italy, and includes Mozzarella, Scarmorza, Provolone, Parmesan, Ciciovell and others (Kosikowski and Mistry, 1988; Fox, 1987). The Alpine cheese originates from the Netherlands, among the Dutch type cheeses and these include Gouda, Edam and others

(Fox, 1987; Sukumar De, 1980). The maturation time of Pasta is 2 - 3 weeks while that of Alpine is 5-6 weeks (Kindstedt, 1993; Barbano, 1994).

2.3.1 General procedure of cheesemaking

The history of cheesemaking has been reviewed by Scott (1986) and Fox (1993). Basically the cheesemaking procedure is similar for most cheeses, (Fig.3) with slight deviations depending on the type of cheese (Sukumar De, 1980; Fox, 1993; Holsinger *et al.*1995). Essentially standard milk (3 - 3.3 % BF) is heated and cooled. A bacterial starter culture is then added to start acid development, coagulation is continued by addition of an enzyme preparation.

Starters are cultures of food grade. Lactic acid bacteria grown in milk or whey which are used to impart certain characteristics and predictable qualities (Fox, 1990; Cogan and Hill, 1993). Mesophillic cheese starter cultures contain lactic acid bacteria: *Lactococcus lactis subsp. cremoris, L. lactis subsp. lactis, Lactobacillus delbrueckii subsp. bulgaricus, Leuconostoc mesenteroides subsp. cremoris.* Homofermentative *lactobacillus* are normally isolated from good quality cheese. Swiss cheese varieties are manufactured with the use of thermophillic *Streptococcus thermophillus* cultures (Fox, 1993). Curdling results from coagulation of the casein and entrapping as much as 90% of the fat. The curd is cut to expel excess moisture, and this can be enhanced by continued development of acidity and by stirring, with a moderate or high degree of heating and in case of hard cheeses by pressing the curd. The curds are shaped, NaCl added and stored for ripening in controlled temperatures and humidity (Sukumar De, 1980, Fox, 1987, 1993; Walstra, 1993).

Steps	Conditions
1. Raw milk	whole milk
↓	
2. Filtration	
↓	
3. Standardization of raw milk	3.0.-3.3 %BF
↓	
4. Pasteurize	72-74 ^{0}C/15 sec
↓	
5. Cool	32-35^{0}C
↓	
6. Addition of starter	1-2%
↓	
7. Incubation	30 min
↓	
8. Addition of rennet	0.05-0.06%
↓	
9. Coagulation	35-40 min
↓	
10 Cutting of coagulum	into cubes
↓	
11. Settle	15 min
↓	
12. Stirring and scalding	32-38^{0}C
↓	
13. Draining whey	
↓	
14. Cheese pressing	1 kg/10 kg weight
↓	
15. NaCl brine	15-20% NaCl
↓	
16 Ripening	controlled temperature and relative humidity

Fig. 3: Flow diagram of a generalized cheese making procedure (Fox, 1993; Holsinger, 1995)

2.3.2 Clotting enzymes and their relation to cheesemaking

The primary function of the milk coagulating enzyme is to effect the coagulation of cheese milk to which it has been added (Visser, 1993; Fox, 1993; Ustunol, 1993; Holsinger *et al.*1995). Many proteolytic enzymes coagulate milk by breaking the Phe_{105} - Met_{106} linkage in κ-casein (Anderson and Andren, 1987; Fox and Stepaniak, 1993, Fox *et al.* 1995). In milk at least 95% of the κ-casein is hydrolysed, and the higher the enzyme concentration used the faster the reaction. Also other proteins in milk are hydrolysed, however, at a much slower rate and of less importance for coagulation (Anderson and Andren, 1990). The coagulation of milk, (Figure 4) can be largely divided into 3 different stages and rennet coagulation has two phases: the primary phase (enzymatic stage) and the secondary phase (non-enzymatic stage) (Davide and Law, 1984; Scott, 1986; Fox, 1987, 1989, 1993; Barbano, 1994).

Primary phase

The enzyme rapidly and specifically cleaves the hydrophilic glycomacropeptide (GMP) part from the κ-casein (κ-CN) molecule located at the periphery of the casein micelles (Dalgleish, 1993; Visser, 1993; Walstra, 1996). In their intact form, these micelles are kept colloidally dispersed in milk by steric and electrostatic repulsion involving negatively charged GMP part of the κ-CN molecule (Walstra, 1990). When this repulsive barrier has been taken away by the enzymatic removal of the GMP part, the micelles become unstable, then at an appropriate temperature of 30 °C the milk starts coagulating under the influence of Ca^{2+} ions in the medium (Anderson and Andren, 1987; Dalgleish, 1982; Visser, 1993).

The secondary phase

As the stabilizing power of κ-CN is destroyed by the enzymatic action the caseinate micelles in milk become progressively more susceptible to clotting in the presence of Ca^{2+} ions. A clot will not form in the absence of calcium ions and renneted micelles are incapable of aggregating until about virtually all of the casein has been destabilized (Webb *et al.* 1974; Fox, 1987; Anderson and Andren, 1987; Dalgleish, 1982). The aggregation rate of renneted micelles is unaffected by the concentration of the rennet or by the size of the milk micelles and will not coagulate when the temperature is below 15 °C (Webb, 1974; Fox, 1987, 1990). Aggregation of micelles is due to absence of repulsive forces and van der Waals attraction may be sufficient to hold the micelles together but a number of other forces may act as well (Scott, 1986; Fox, 1987, 1993; Holsinger, 1995).

The final stage

The final stage in the coagulation is called syneresis. The syneresis, implying the expelling of whey from the gel, is induced by breaking down the gel and applying pressure so that a number of new and more linkages are formed. As a result, both the casein micelles and the pores between them contract and finally, the syneresis is stopped sterically owing to the position of the fat globules (Anderson and Andren, 1987; Dalgleish, 1993; Holsinger, 1995).

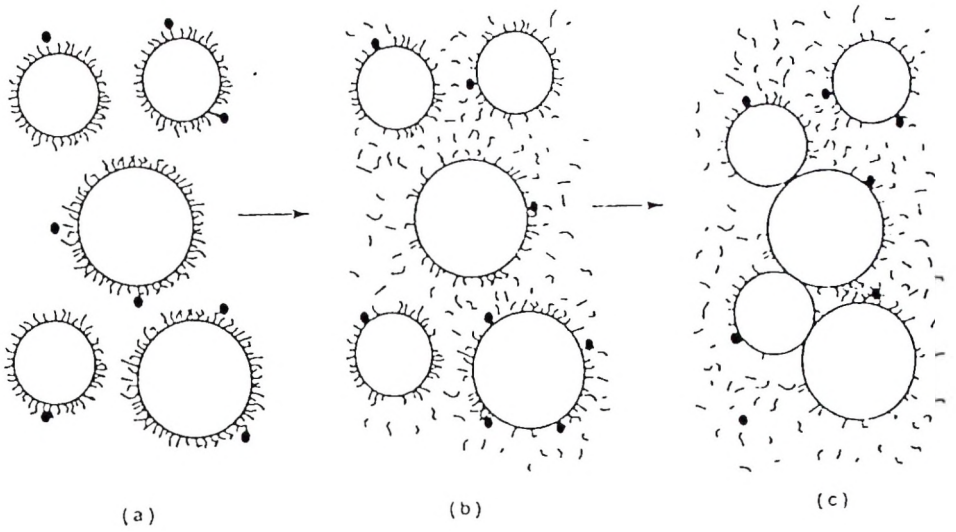


Figure 4: Schematic diagram of the attack by chymosin (filled circles) on casein micelles.

Three different points in the reaction are illustrated. In (a) the κ-casein (κ-CN) coat of the micelles is intact and chymosin have just been added; (b) some time later the κ-casein has been hydrolysed and a portion of a GMP is in solution but sufficient remains to prevent aggregation; (c) at a later time still, nearly all of the κ-CN has been hydrolysed and the micelles have started to aggregate (Anderson and Andren, 1987; Dalgleish, 1993; Visser, 1993)

2.3.3 Factors which affect rennet action

Several factors affect the activity of rennet but the four major ones include temperature, acidity, calcium ion concentration in milk (Lenoir and Schneid, (1987) and inhibitory substances (Hill, 1995). Lenoir and Schneid (1987 reported that low Ca^{++} in milk lead to slow coagulation and soft gel which tends to break up into cheese fines. Lenoir and Schneid (1987) reported that milk with genetic variant B of κ-CN is more sensitive to coagulation than with κ-CN of genetic variant A. Sukumar De (1980) showed that chymosin is almost inactive below 20 °C with an optimum range of 30 – 48 °C. Rate of clotting increases rapidly with small increases in pH above 6.0. However, above pH 7.2 - 7.4 chymosin undergo autolysis (Visser, 1993). Calcium ions content of milk affect

the 2nd phase (Sukumar De, 1980; Fox *et al.*1993, 1995) while the 1st phase is not affected by calcium. Inhibitory substances, mainly colloidal substances, such as albumin, serum peptones and others (Davis, 1965; Sukumar De, 1980; Scott, 1986; Hill, 1995) and mastitic milk retard or may even prevent enzymatic activity due to the alkalinity of the milk which may reach pH 7-7.2. Martley *et al.* (1993) reported that curd may lack strength, shatter, from homogenised milk which lead to losses of fine particles into the whey and the final product may have a poor texture characteristic (Hill, 1995).

a) Loss of constituents in the whey

Loss of contituents into the whey dependent on the type of cheese, the ease and frequency of agitation of milk and curd and the temperature involved. The type of milk clotting enzyme dictates the loss of constituents into the whey (Barbano *et al.* 1993 and Perlmann and Kelly, 1993). Adult bovine pepsin causes higher fat losses in whey (Barbano *et al.*1992). Rennet gave a higher cheese yield in comparison to *M. miehei* and *M. pusillus* preparatios. On the other hand, recombination produced rennet and calf rennet had identical cheese yield efficiencies but adult bovine pepsin had lower cheese yield efficiency (Barbano *et al.*1994; Farmakalids, 1995). Lenoir and Schneid (1987) reported that when coagulation is slow the curds becomes soft and breaks more easily into fine particles causing loss of BF and TN in whey.

b) Heat treatment of milk

Pasteurisation increase the yield of cheese if the milk is heated to an extent that serum proteins are denatured and also increasingly diminishes the syneresis rate of renneted

milk (Walstra, 1993). Thus the increase is attributed both to the aggregation of whey proteins with casein micelles and to the enveloping effect of the precipitated protein on the fat globules (Fox, 1987, 1993).

2.3.4 Cheese ripening

Ripening is arbitrarily referred to methods and conditions (temperature, humidity, and others and treatment) from manufacture to marketing of cheese (Webb *et al.* 1974). The ripening, maturing or curing of cheese refers to the storage of cheese for 2 to 3 months or longer at a given low temperature (0 - 16° C) (Fox, 1987).

Table 3: Cheese curing systems

Particulars	Cold curing	Warm curing
Temperature	0- 4 ^{0} C(32-40 ^{0} F)	10-16 ^{0} C (50-60^0 F)
	average 1.5 ^{0}C/ 35 ^{0} F	12.5 ^{0}C/ 55 ^{0} F
Humidity	75%	85%
Duration	2-12 months	2 weeks - 2months
Quality of cheese	Mild flavour	Sharp flavour
	Bacterial defects minimum	Bacterial defects exaggerate

Sukumar De, (1980)

Fox *et al.* (1993) reported that four or possibly five agents are involved in ripening of cheese. These are: rennet or rennet substitute (chymosin, pepsin or microbial proteinases); indigenous milk enzymes which are particularly important in raw milk cheese; starter bacteria with their enzymes which are released after the cell have died and lysed; enzymes from secondary starters; and from non-starter bacteria (those which either survive pasteurization or gain access to the pasteurized milk).

Webb *et al.* (1974); Fox (1993, 1995) and McSweeney and Fox (1997) reported vital changes taking place during the ripening process which include both physical and chemical changes. The physical changes are essentially centred on the moisture content of cheese (Scott, 1986) and its relation to the scalding temperatures. For example Emmental cheese scalded from 49 to 56°C becomes a rubbery cheese when mature (Davis, 1965; Steffen *et al.*1993).

Biochemical changes include proteolysis, lactose fermentation and lipolysis (Davis, 1965; Davies and Law, 1984 and Fox, 1987). The secondary flora of cheese include lactobacilli, micrococci, brevibacteria, pediococci and dairy propionic bacteria non

starter lactic acid bacteria (NSLAB). Micrococci are a major portion of the milk flora itself, some of which are thermoduric and hence survive in pasteurised milk. Micrococci and pediococci improve flavour. Their beneficial role has been related to their proteolytic, lipolytic and esterolytic activities and their metabolites produced during ripening (Bhowmik *et al.* 1990; Fox and Stepaniak, 1993; Steele, 1995).

a) Proteolysis

Proteolysis is the most complex biochemical process that occurs during maturation of the majority of cheese types (Scott, 1986; Fox, 1993; Innocente, 1997 and McSweeney and Fox, 1997). Proteolysis is vital in impacting flavour and texture to cheese and Fox (1993) reported at least four ways by which proteolysis in cheese contribute to cheese ripening: a direct contribution of amino acids and peptides to the desirable flavour of cheese and some which may cause off-flavours especially bitter or indirectly via catabolism of amino acids to amines, acids, thiols, thioesters; greater release of sapid compounds during ripening; changes in pH via the formation of NH_3; changes in the texture arising from breakdown of protein network; increase in pH and greater water binding by the newly formed amino and carboxyl groups. Proteolysis has been divided into three phases: proteolysis in milk before cheese manufacture; the enzymatically induced coagulation of the milk and proteolysis during cheese ripening. Proteolysis is vital for flavour and texture of cheese (Samples *et al.* 1984; Fox, 1987; McSweeney and Fox, 1995).

Protein digestion is minimal at commencement of ripening; only about 4% of the total protein is soluble in water. The soluble protein increases with ripening time. In a well ripened hard cheese the soluble nitrogen increases to about one-third of the total protein

(Fox, 1993) while in mould ripened soft cheese it may increase to two-thirds and even higher. The state and extent of proteolysis is used as a measure of ripening (Walstra *et al.* 1993 and Fox *et al.* 1993, 1995 and Pecoran *et al.* 1997). Innocente (1997) reported that traditionally in semi-hard cheese, the development of proteolysis is followed by an increase of water soluble nitrogen as percent of total nitrogen. Webb *et al.* (1974); Fox *et al.* 1995); McSweeney and Fox, 1995, 1997) reported that ripening occur in two stages:

- Hydrolysis of protein to peptides and proteoses peptones
- Hydrolysis to lower molecular weight peptides and amino acids.

Webb *et al.* (1974) reported that during a ripening period of 1 year, water soluble nitrogen in cheddar cheese increased to 44.7% of total nitrogen, amino nitrogen increased to 28.4% of the total and ammonia nitrogen to 5.4% of the total while the proportion of peptides and peptones proteoses initially increased and then decreased. The decrease being attributed to the conversion of the proteoses peptones to amino acids (Webb *et al.*1974; Gripon *et al.*1991; McSweeney, 1997).

Proteolysis in Dutch-type cheese varieties is mainly due to the action of calf rennet enzymes, enzymes of starter bacteria and to a small extent milk proteinases (Fox, 1987 and Walstra *et al.*1993). Lactobacilli are highly proteolytic while *S. thermophilus* is less proteolytic (Rajagopal *et al.* 1990). In hard Italian cheese the level of proteolysis is similar to that in other hard/semi-hard bacterially ripened cheese (Fox, 1987 and Barbano and Rasmussen, 1994).

b) Lipolysis

Lipid or fat breakdown in cheese contributes mainly in flavour development (Hill, 1995 and Montorfano *et al.* 1996). The lipolytic enzymes release fatty acids by hydrolysis of the fat which is vital in the ripening of many cheese varieties (Fox, 1987 and Walstra *et al.* 1993). Lipases occur in normal milk, in micro-organisms involved in the ripening process and in some rennet preparations (Fox, 1987, 1993). In the manufacture of certain Italian cheeses, rennet paste has been found to produce more flavour than rennet extract since the paste contains lipolytic as well as proteolytic enzymes in addition to chymosin and pepsin (Steffen *et al.* 1993 and Montorfano *et al.* 1996). The content of free fatty acids in hard cheese is 700 - 1481 mg/kg, in Italian cheese 6700 mg/kg and in mould cheese (Roqueford type) 32,000 mg/kg (Fox *et al.*1995).

c) Fermentation of lactose and citric acid

Lactose fermentation occurs primarily during cheese manufacture and in the first stages of cheese ripening (Walstra *et al.* 1993, 1995; Hill, 1995)) resulting in production of lactic acid but also propionic acid and acetate as well as number of volatile flavour compounds and carbondioxide responsible for eye formation in cheese. Formation of lactic acid is of great importance in the preservation of cheese, proper manufacture of cheese and the ripening of cheese (Fox, 1987 and Walstra *et al.* 1993) including the ripening of cheese in an acid medium. Lactic acid suppresses undesirable micro-organisms (Webb *et al.* 1974). Citric acid fermentation which lead to CO_2 production is of particular importance to flavour and eye formation in Dutch-type varieties (Fox, 1987; Walstra *et al.*1993). Fox, (1993) reported that acid production at appropriate rate

and time is the key step in the manufacture of good quality cheese. Acid affect several aspects of cheese manufacture such as: coagulant activity during coagulation; denaturation and retention of the coagulant in the curd during manufacture and hence the level of the residual coagulant in the curd, which influences the rate of proteolysis and may affect cheese quality; curd strength, which influences cheese yield; gel syneresis which controls the cheese moisture and hence regulates the growth of bacteria and the activity of enzyme in the cheese (Fox, 1987; Holsinger, 1995). Consequently it strongly influences the rate and pattern of ripening and the quality of the finished cheese. The rate of pH decrease determines the extent of dissolution of the colloidal calcium phosphate which modifies the susceptibility of the caseins to proteolysis during the manufacture and influences the rheological properties of the cheese. Acidification controls the growth of many species of non-starter bacteria in cheese especially pathogens, food poisoning and gas-producing microoganisms. Many starter cultures also produce bacteriocins that also restrict or inhibit the growth of non-starter micro-orgasms (Fox, 1993; Zottola and Smith, 1993).

2.3.5 Factors affecting cheese ripening/maturation

a) Temperature

Rate of ripening of hard cheeses depends on temperature of ripening (Webb *et al.* 1974). High temperatures although accelerating ripening, leads to formation of structure and texture defects. According to Fox (1987) high temperatures of 15- 20° C favour proteolysis (Steffen *et al.* 1993) and formation of bitter flavours (Lee *et al.* 1990).

b) Amount of clotting enzyme

The amount of enzyme is directly proportional to proteolysis, lipolysis and hence extent of ripening (Walstra *et al.*1993; Fox *et al.*1995). The more enzymatic concentration, the greater is the extent of proteolysis (Webb *et al.* 1974; Fox, 1987). About 6 % of the chymosin is retained in cheese curd and plays a major role in the initial proteolysis in cheese (Fox, 1989, 1993, 1995). Limited proteolysis is one of the characteristic to be considered in the selection of proteinases for use as rennet substitute (Fox, 1989 and Hill, 1995)

c) Moisture content

A high moisture content in cheese leads to extensive degradation of protein (Fox, 1987; Webb *et al.* 1974). Coating cheese with paraffin and use of packing materials such as paper, plastics, aluminium and metals reduce loss of moisture, though the ripening process may be longer (Stehle, 1987).

d) NaCl concentration

NaCl, (NaCl) have major effects on hydrolysis of β-casein by chymosin in solution (Fox and Walley, 1971) and in cheese (Kelly, 1993) and may be a factor in the development of bitterness. Kelly, (1993) found that formation of bitter β-casein f193-209 primarily produced by chymosin could be inhibited by increasing NaCl concentration. Various NaCl concentrations in cheese retard the ripening processes. A low sodium chloride content favours proteolysis and the activity of milk lipase is reduced by the sodium chloride in the cheese (Fox, 1987; Guiney and Fox, 1993). NaCl improves flavour during ripening and the desiccating effect increases firmness

especially with respect to rind formation, so that cheese is subjected to less damage in handling. Too little NaCl causes a pasty body, abnormal ripening and increased shrinkage in curing, while too much causes a dry brittle body and cracking of the rind (Ling, 1963).

According to Fox (1987), the main factor that influences the rate of proteolysis, appear to be NaCl in moisture. A cheese with a low NaCl in moisture value has a higher rate of proteolysis than a cheese with high NaCl in moisture, Webb *et al.* (1974); Guinee and Fox (1993) states that NaCl decreases the rate of formation of protein decomposition products. High NaCl content in cheese causes loss of moisture from the curd and at the same time is removed, Guiney and Fox (1993).

2.3.6 Flavour and texture of cheese

Flavour is defined as the complex sensation comprising aroma, taste and texture (Fox, 1987). In spite of intensive research for many years (Fox *et al.* 1995) only limited information is available on the chemistry of the flavour of most cheese varieties, and the flavour of none is characterised sufficiently to permit its reproduction by mixtures of pure compounds (Fox *et al.* 1995). Flavour has three basic components olfactory, gurtatory and tactual which are concerned with odour, taste and physical feel of a flavour stimulus. Taste is concerned with sensation detected in the mouth especially on the tongue, that is sweetness, sourness, saltness and bitterness. The tactual part of flavour is the way the substance feels in the mouth. Thus, sensations of smoothness, graininess, tenderness, chalkiness are tactual flavour responses (Jenness and Patton, 1959). Flavour can be categorised on the basis of distillation, namely the non volatile portion and the volatile portion (Webb *et al.*1974; McSweeney and Fox, 1993). The

non- volatile portion is responsible for taste and contains the amino acids, non-volatile acids, amines, minerals and salts while the volatile part which is responsible for aroma consists of fatty acids, aldehydes, ketones, alkaloids, amines, esters, hydrogen sulphide and sulphides (Fox *et al.* 1995). According to Jennes and Patton (1959) flavour may logically be divided into two groups namely the normal or natural flavours and the off-flavours. A substantial part of the research in this area has been on cheddar cheese (Lawrence *et al.*1993; Oslon, 1990; Steele and Unlu, 1992; Urbach, 1993 and Fox *et al.*1995)

i) Cheese flavour

No single one or a simple combination of flavour producing substances such as peptides, fatty acids, aldehydes, esters and others possess typical cheese flavour (Webb *et al.* 1974). The aroma compounds in Dutch-type cheese are the breakdown products of lactose and citric acid (lactic acid, diacetyl, carbon dioxide and others of paracasein (peptides and amino acids); and of lipids (free fatty acids) which are essential for the flavour (Fox, 1987; Walstra *et al* 1993). Essentially typical cheese flavours result from a correct balance or the blending of a variety of specific individual substances in proper proportions (Webb *et al.* 1974; Fox, 1987 and Fox *et al.*1995). This has been termed as the "Component Balance," Mocquot, (FAO, 1958) as quoted by Webb *et al.* 1974). Shifts in the balance cause shifts in quality or the intensity of the cheese flavour. A small percentage of diacetyl (0.05mg or less per 100gm) contributes to a desired characteristic Cheddar flavour while a high content may produce flavour defects (Webb *et al.* 1974). Thus a given cheese derives its characteristic flavour from a mixture of compounds (Webb *et al.* 1974; Fox, 1987; and Fox *et al.* 1995).

ii) Carbohydrates in relation to flavour

Lactic acid brings about the refreshing acid taste, particularly noticeable in young cheese (Fox, 1987; Davies *et al.*1984) and excess lactic acid renders cheese sour. Lactic acid built from lactose fermentation is more vital as an antimicrobial agent (Davies *et al.*1984; Fox, 1987 and Webb *et al.* 1974).

Lactic acid acts as an aid for production of flavour compounds in that it allows development of full flavoured cheese by ensuring that enzymatic reactions proceed slowly (Davies *et al.*1984). Also it contributes to the redox potential of cheese ensuring that flavourful sulphur compounds remain in their reduced form (Davies and Law, 1984). Metabolism of citrate by *L. lactis sbsp. lactis, biovariant diacetylactis* and *Leuec. mesenteroides subsp. cremoris* produces diacetyl and carbon dioxide (Davies and Law, 1984).

iii) Lipids as flavour attributes

Davies and Law (1984) stated that triglycerides from milk are the most vital lipid class in cheese and phosphor-lipids present do not contribute to flavour of cheeses. Lawrence *et al.* (1993) and Fox *et al.* 1995) stated that fat must be there for perception of flavour, and it is well accepted that cheese from skim milk does not develop a characteristic flavour. Italian cheeses, (Roman, Parmesan and Provolone) are entirely dependent for their distinctive flavour on free fatty acids released (Davies and Law, 1984; Fox, 1987 and Steffen *et al.*1993). In addition, free fatty acids act as substrates for oxidation to methyl ketones which are also flavour compounds (Davies and Law, 1984). Substituting vegetable oil or mineral oil for milk fat still results in a degree of flavour in cheddar cheese (Lawrence *et al.* 1993).

iv) Protein as flavour attributes

Proteolysis is also crucial in flavour formation (Fox, 1987). Amino acids and peptides are the proteolytic products which contribute to the basic flavour of cheese (Fox, 1987 and Lawrence *et al.*1993). According to McGugan, (1979) as quoted by Davies and Law (1984) the water-soluble fraction of cheese (amino acids and peptides) is the most prominent in relation to the intensity of cheese flavour. General flavour characteristics common to many surface ripened varieties of cheese have been attributed to 3-methyl-1-butanol, phenylethanol and phenol (Davies and Law, 1984 and Lawrence *et al.* 1993).

2.3.7 Flavour defects

In addition to contributing to cheese flavour, products of proteolysis are attributed to flavour defects such as bitterness (Webb *et al.* 1974; Davies and Law, 1984 and Steffen *et al.* 1993). Rancid flavour is a defect arising from the spoilage of free fatty acids (Webb *et al.* 1974 and Fox, 1987). Certain peptides are the source of bitterness in cheese (Fox *et al.* 1995). The bitterness in cheeses has been ascribed to high proteolytic activity of rennet at low pH and reduced ability of bacterial proteinases and peptidases to convert polypeptides (Davies and Law, 1984; Kelly, 1993). Fox and Stepaniak (1993) demonstrated the production of bitter peptide from αs1-CN f165-199 by lactococcal endopeptidase (LEP III-I). The phenolic flavour in Gouda cheese was due to p-cresol produced by a special group of lactobacilli (Webb *et al.* 1974). Other flavour defects include fruity flavour in cheddar due to ethanol, ethylcaproate and ethylbutyrate. A catty flavour called Ribes defect is an odour of feline urine identified as a 6-carbon monounsaturated ketone (Webb *et al.* 1974). Various micro-organisms cause flavour defects, predominantly in cheese made from raw milk, such as *L. lactis*

sub. lactis var *maltigenes* (burned flavour), *S. faecalis* var *malodoratus* (hydrogen sulphide flavour), and yeast (yeasty, fruity flavour) (Fox *et al.*1995).

a) Cheese texture

Texture describes the tissue structure of substances which is characterized by detailed subdivisions of the tongue and pressure sensation perceived through biting, chewing and swallowing (Watts *et al.* 1989). Cheese texture may be influence by the processing conditions in the manufacture of cheese (Davies and Law, 1984 and Walstra *et al* 1993). Crumbiness of cheese indicates the extent of curd fusion while firmness is dependent on extent of proteolysis during ripening (Davies and Law, 1984 and Walstra *et al.*1993). The consistency of the Dutch-type cheeses is affected by moisture content, extent of proteolysis, pH, sodium chloride and fat content (Fox, 1987 and Walstra *et al.*1993). An important textural characteristic in Dutch-type cheeses is the number, size and shape of holes (Fox, 1987). Textural defects may arise from activities of deteriorative micro-organisms such as coliform bacteria resulting in early blowing with off-flavours (yeasty and gassy flavours) (Fox, 1987; Walstra *et al.* 1993). Descriptions used for cheese texture include density tenderness, granular structure, fragility.

During maturation, several changes occur to the texture of the cheese: structure and composition becomes more uniform, particularly in the early stages due to further fusion of curd grains and reduction of NaCl, moisture and pH gradients. Cheese looses water by evaporation and ongoing syneresis especially near the rind and due to proteolysis; maturation which primarily implies breakdown of the paracaseinate network, also causes a slight increase in pH and gas is formed (Walstra *et al.* 1993)

2.3.8 Microoganisms in cheese in addition to starters

During cheesemaking, starter cultures are added intentionally in cheese to enhance flavour and improve texture qualities of the final product. Also some non-starter lactic acid bacteria (NSLAB) enter as a result of contamination with milk supply or processing equipments. Their main source is raw milk and Martely and Crow, (1993) reported that most of them are killed during pasteurisation and scalding but some recontaminate the cheese. The dominance of the lactic starter culture at this stage leads to high total counts in cheese. During brining, some microbes are inhibited from growing, and those that survive brining are further restricted in growth due to low pH, low temperature of brine and low moisture as cheese mature. Steele (1995) reported a decrease from 10^8 to 10^7 cfu/g after 14 days in cheddar cheese. So as the cheese ripens more viable starters and adjuncts decrease as they die and lyse in cheese matrix and the lysis and autolysis result in the release of intracellular components such as sugars, nucleic acids, serving as nutrients for growth of NSLAB (Steele, 1995; Martely and Crow, 1993; Walstra *et al.*1993). The rate of autolysis and nutritional quality of the autolysate differ from strain to strain of starter cultures and adjuncts have a significant impact on growth of NSLAB which are initially present in small amounts but reach high numbers during long cheese ripening period (McSweeney *et al.* 1993). They have a significant influence on flavour development of cheese (Steele, 1995; Martely and Crow, 1993). NSLAB consists mainly of lactobacilli, leuconostocs, streptococci and micrococci which are heat resistant and most of them are initially high in number but die as cheese ripening continue except lactobacilli which are the only lactic acid bacteria in mature cheese and can multiply and dominate in mature cheese, (Martely and Crow, 1993; McSweeney *et al.*1993; Steele, 1995).

Deteriorative micro-oganisms will develop in cheese and are mainly determined by the microbial composition of milk, any contamination during cheesemaking, and composition of cheese during curing conditions (Zottola and Smith, 1993 and Walstra *et al.*1993). Most undesirable microbes are killed during pasteurisation but the extent of defects may differ greatly between cheese made from raw or from pasteurised milk. In spite of high hygienic standard, contamination of milk and cheese curd with undesired microbes cannot be fully prevented (Chapman and Sharpe, 1981; Walstra *et al.*1993). Coliforms grow during the early stages of cheesemaking and can multiply very rapidly during the first few hours when the conditions such as pH, and temperature are favourable. Excess growth leads to development of off-flavours (yeasty, gassy and unclean flavour) and showing early blowing of cheese due to CO_2 and H_2S produced. Hygienic conditions and rapid acidification of curd to reduce the pH and amount of lactose in cheese are factors of great importance in preventing coliforms. Also use of nitrates prevent the development of *Clostridium botulinum* and accumulation of hydrogen and butyric acid (Zottola and Smith, 1993).

Lactobacilli are NaCl tolerant and can induce flavour and texture defects in mature cheese. Common ones isolated from high quality cheese are *L. plantarum*, *L. casei* and *L. brevis* (Steele, 1995) which grow slowly but at maturity can go up to 2×10^7 and 10^8 cfu/g cheese causing gassy and putrid flavour and excessive open texture (McSweeney *et al.*1993). Low strength brine < 15 % NaCl gives rise to growth of NaCl tolerant lactobacilli as they penetrate the cheese rind. Other types are thermoresistant streptococci which mainly originate from milk. Propionic acid bacteria have considerable growth in Swiss-type cheeses causing sweet and nutty taste and very open texture due to excessive gas formation. Higher temperatures and pH 5.1-5.2 favour

their growth (Zottola and Smith, 1993 and Chapman and Sharpe, 1993). Abundant growth of yeasts and moulds on cheese surfaces may cause slimy rind, part coloured or pink appearance on cheese surfaces (Chapman and Sharpe, 1981). Growth is favoured by insufficient acidification of cheese, low NaCl in brine, high pH, inadequate drying and insufficient cleanliness of shelves in the curing room. Excessive growth cause toxins in cheese and H_2S flavour, fruity flavour and the cheese becomes rancid (Zottola and Smith, 1993). Post pasteurisation contamination, faulty pasteurisation and faulty manufacturing practices are main contributors to gross contamination in cheese (Chapman and Sharpe, 1981; Walstra *et al.* 1993; Martely and Crow, 1993; Steele, 1995).

2.4 Conclusion from literature review

From the literature review, it can be concluded that in order to obtain bovine pepsin extract from adult cattle abomasa, conditions such as ionic strength and pH of the extraction solution, maceration time and temperature, activation pH and time of the crude extract, clearing and storage conditions of the extract affects the milk clotting activity of the extract. Therefore these conditions must be investigated before the actual bovine pepsin extraction.

For bovine pepsin to be characterized, it must be purified first. There are many methods used in separation and purification of bovine pepsin but ion exchange chromatography is the most common and simple way of purifying crude pepsin extract to homogenity. Measurement of MCA, determination of protein concentration in chromatographic fractions, use of electrophoresis, behaviour of chromatography,

molecular mass determinations and amino acid sequence are some of the ways used in quantifying the purification steps and characterization of the purified pepsin extract

In order to assess the quality of bovine pepsin in cheesemaking: quality of cheese milk, cheesemaking procedures, cheese ripening conditions and sensory characteristics of cheese must be well controlled and investigated.

CHAPTER 3.0

MATERIALS AND METHODS

3.1 Experiment 1: Extraction of bovine pepsin

3.1.1 Introduction

Essentially, extraction of crude bovine pepsin is an attempt to simulate what is taking place inside the animal abomasum and bring the enzyme is solution (Whitaker, 1972). This is achieved by providing optimum conditions which will favour the release and conversion of pepsinogen to pepsin without loss in activity.

Many researchers have extracted pepsin enzymes using different conditions depending on available facilities, financial position and use of the enzyme after extraction. In this study bovine pepsin was extracted based on conditions which would utilize locally available, low cost and easy to use materials in Tanzania and at the same time optimize milk clotting activity (MCA). The conditions which were tested were: the location of the abomasum with high pepsin concentration; extraction of pepsin from fresh versus dry abomasa; drying under direct sunshine or shade or forced air oven; extraction solutions of different pH levels; extraction temperatures; ionic strength of extracting solutions; maceration periods; activation pH and times. Furthermore, clarification methods and storage temperatures were also studied. The conditions which lead to maximum MCA were then used to extract pepsin using locally available solutions in Tanzania.

The main aim of this study was to develop a suitable method of preparing crude pepsin extract from adult cattle abomasa using locally available materials.

The specific objectives were:

1. To determine extraction and storage conditions which can lead to optimum MCA of pepsin extracted by dilute HCl
2. To compare the extraction efficiency of three different solutions of locally available materials in extracting pepsin using optimum conditions established under objective 1

3.1.2 Experimental approach of the study.

In this study, determination of optimum pepsin extraction conditions was done first, and then the optimum conditions were used to extract pepsin using different solutions

Collection of abomasa for pepsin extraction

Abomasa were collected from Morogoro town abattoir immediately after slaughter in the early morning hours (5-6 am). In the laboratory they were immediately washed with running water, fatty tissues trimmed away, rinsed with 5% brine solution for preservation and frozen immediately or dried under shade or direct sunshine.

Preparation of extraction solution

The extraction solution was prepared according to the method described by Hagyard and Davey (1972) by mixing one liter distilled water with 0.5 ml HCl of 37 % concentration drop wise until the pH reached 1.4 (about 11 drops) plus 100 g NaCl (10%), plus 40 g $CaCl_2$ (4 %) and 2 g sodium benzoate (0.2%) as a preservative.

Determination of MCA

The MCA of the enzyme extract was determined by the Roll tube method according to IDF Standard 157:1992

A hundred and ten grams of low heat dried skim milk powder obtained from Tanzania Dairies Limited (TDL) was reconstituted in one liter of distilled water containing 0.05 % $CaCl_2$. Results were reported in minutes or pepsin units (PU)/ml.

3.1.3 Optimization of extraction conditions for high MCA

Comparison of pepsin concentration in the fundic and pyloric regions of abomasa

Seven abomasa were cut dorsally and sun dried (temperature 25-28° C) to constant weights attained in four days. The abomasa fundic and pyloric regions were easily distinguished by the presence of mucosa tissues in the fundic and their absence in the pyloric regions. After separating these parts, each part was cut into small pieces (0.5-1 cm wide). Duplicate samples of 10 g were mixed with100 ml extraction solution, macerated at room temperature for 3 days. The crude extract was activated with 0.1 N HCl for 60 min and clarified by adding 1 g $Na_2HPO_4.H_2O$ for 20 ml extract to rise pH to 5.4. MCA was determined.

Effect of extracting pepsin from dried versus fresh abomasa

Seven fundic regions of the abomasa were slit into two equal portions. One part was dried under direct sunshine to constant weight and cut into small pieces. The amount of water lost was calculated and the same amount of water was added to reconstitute the tissue before enzyme extraction. Other portion, (the fresh abomasa) was cut into small pieces and ground in a waring blender in an extraction solution. For example: from one

part of the fundic region, 127 g was dried under direct sun to constant weight of 30 g, cut into small cubes 0.5 cm, mixed with 97 ml water lost during drying plus 381 ml extraction solution with 2 g sodium benzoate. The other 127g of fresh fundic abomasa was cut into pieces, blended, with 381 ml extraction solution plus 2 g sodium benzoate. All the portions were macerated for the same period of 3 days, activated, clarified and MCA determined.

Effect of drying methods on the fundic region of the abomasa

Three fresh abomasa were each divided dorsally into three parts and each part was dried to constant weight under direct sunshine (25-28° C), in the forced air oven (30° C) and under the shade. Each part was cut into small pieces and duplicate samples of 10 g were mixed with 100 ml extraction solution, macerated, activated, clarified and MCA determined. Extracts were also determined for dry matter content as described by Egan *et al.* (1981) and cultured for Standard plate count (SPC) as described by IDF standard 100B: 1991.

Determination of the optimum NaCl concentration for pepsin extraction

Duplicate samples of sun dried pieces of fundic abomasa each weighing 10 g were put into 10 flasks of 250 ml and mixed with 100 ml extraction solution of varying NaCl percentage: 5, 10, 15, 20 and 25 g. The mixtures were macerated for 3 days, activated, clarified and MCA determined.

e) Determination of optimum extraction temperature

Duplicate samples of sun dried pieces of fundic abomasa each weighing 10 grams were put into 6 flasks of 250 ml and mixed with 100 ml extraction solution and macerated at 37^0 C, room temperature 24 -28^0 C and incubator of 28-30^0 C for 3 days. The extracts were activated, clarified and MCA determined.

Determination of the optimum extraction pH and time on MCA

To the 10 grams of sun dried pieces of fundic regions of the abomasa put into 12 flasks of 250 ml, 100 ml of extraction solution with pH of 1, 2, 3, 4, 5, and 6 were added into duplicate flask. They were macerated for 6 days at room temperature, activated for 1 hour, clarified and MCA was determined at intervals of 1, 2, 3, 4, 5, 6 days.

Determination of the optimum activation pH and time

One hundred grams of sun dried pieces of abomasa were put into one litter of extraction solution. The mixture was macerated at room temperature for 3 days. Its initial pH was 4.47. The 100 ml extract was put into 8 flasks of 250 ml for activating the solutions to the pH of 1, 2, 3, and 4 respectively using 0.1 N HCl. When the activation pH was attained, 1 ml of the solution was drawn out and used to determine the MCA at 10 min intervals. This was continued until the MCA stopped increasing and the time taken to clot milk was recorded as the optimum for activation. The procedure was repeated for activation pH of 2, 3, and 4.

The effect of clarification methods

After maceration and filtering, the crude extract still contained much fine tissues making it turbid and with reduced shelf life of the extract. Clarification therefore was necessary. The crude extract left under 3.1.3 was used in the clarification process as follows:

1. Centrifugation: MCA of the crude extract was determined and then duplicate samples of 5 ml of crude extract was put into three sets of 10 ml test tubes and centrifuged at 1000 centrifugal force (*g)* for 10, 20 and 30 min. This procedure was repeated for centrifugal speeds of 2000, 3000 and 4000 *g* for 10, 20, and 30 minutes respectively. The clear solutions were each determined for their MCA.

2. The remaining portion of the crude extract was divided into 50 ml volumes into 8 beakers of 250 ml and clarified by adding concentrated Na_2HPo_4, $Na_2HPO_4.H_2O$, Na_2HCo_3 and NaOH in duplicate beakers. These chemicals were added to the extracts gradually, stirred with magnetic stirrer until pH rose to pH 5.4 whereby the extract started to flocculate. The extract was left to settle during which the clear solution separated on the top and the precipitate settled at the bottom of the beaker. MCA of the filtrates was determined. Since under most conditions in Tanzania centrifuges and chemicals are very expensive or not available, other simple ways of clarification were investigated. These were filtering using Whatman filter paper No 4, gravity settling overnight and compared them with centrifugation at 3000 g for 30 min. MCA was determined before and after clearing the extracts.

Effect of different storage temperatures

Duplicate samples were stored at three temperature regimes: room temperature (25-28 ^{0}C), refrigeration (0-5 ^{0}C and freezer temperature (-18 to -20 ^{0}C) MCA was determined before and after 6 weeks of storage.

The effect of interaction of different extraction conditions

Ten gm of sun dried pieces of abomasa were put into 108 flasks of 250 ml and 100 ml of extraction solution with pH 1, 2, 3, 4, 5 and 6 to which NaCl concentration levels of 0, 10, 20 % were added. Duplicate sample of each pH and its three NaCl levels were macerated at three temperature regimes of 0-5 ^{0}C, 24 – 28 ^{o}C and 37 ^{o}C for six days. At day 1, 3 and 6 crude extract of 20 ml was drawn from each flask, filtered, centrifuged and MCA was determined. Design used was: 6 pH x 3 NaCl x 3 temperature x 3 days (6 x 3 x 3 x 3 factorial design).

The statistical model :

$$Y_{ijkl} = \mu + P_i + S_j + T_k + D_l + (PxS)_{ij} + (SxT)_{kl} + E_{ijkl}$$

Y_{ijkl} = Effect of pepsin activity μ = the overall mean

P_l = Effect of pH on pepsin activity S_j = Effect of pH on pepsin activity

T_k = Effect of pH on pepsin activity D_l = Effect of pH on pepsin activity

$(PxS)_{ij}$ = Effect of pH and NaCl on pepsin activity

$(SxT)_{kl}$ = Effect of NaCl and temperature on pepsin activity

E_{ijkl} = Residual effect

3.1.4 Comparison of different extraction solutions on the crude pepsin

In section 3.1.2 extraction of pepsin required the use of dilute HCl which is expensive and require careful handling. A study was therefore carried out to use cheap and easily available local materials. The three solutions compared were: fresh lemon juice (pH 2.4 - 2.6), commercial vinegar (pH approximately 2.4) and HCl (pH 2.4, control) The process involved in the extraction is summarized in Figure 5.

3.1.4 Comparison of different extraction solutions on the crude pepsin

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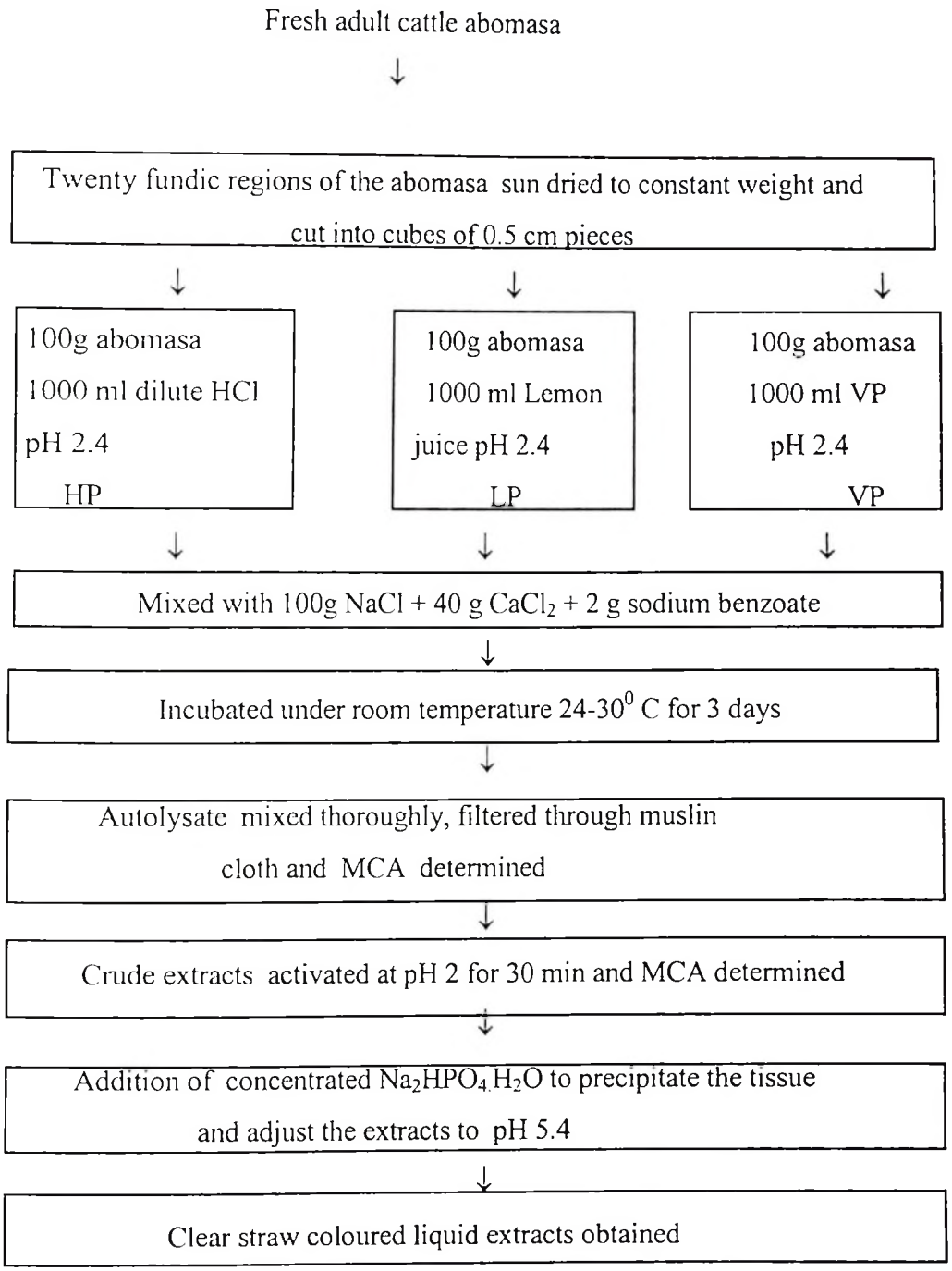


Figure 5: Summary of steps followed in pepsin extraction using different solutions

Without further purification or concentration, the extracts were stored in glass bottles at 5°C for different analyses. The commercial Norwegian liquid chymosin/pepsin (25/75) (STD) was used as a control and MCA was determined.

The Physical characteristics of the extracts

Colour of the extracts

The three extracts were put in beakers and visually observed for their colour, smell and taste comparing them with the STD. Colour was described as yellow or brown yellow or brown or dark brown, smell also was described, taste was described as slight sour or sour or very sour. Turbidity as slight turbid or turbid or very turbid.

Determination of pH and acidity of the extracts

The pH and acidity of the extracts were determined as described by Egan *et al.* (1981).

Determination of dry matter of extracts

The dry matter (DM) of the extracts were measured by using conventional method of dry matter determination as described by Egan *et al.* (1981).

Determination of protein concentration of extracts

Determination of protein concentration of the extracts was done by using spectrophotometer set at a wave length of 280 nm.

Standard solution were prepared using commercial pepsin by dissolving 1, 2, 3, 4 and 5 g into 100 ml of distilled water. One part from each solution was diluted 5 times. The

absorbence of the standard solutions were read on the spectrophotometer and their known concentration were used to make a standard curve.

Duplicate samples of extracts (one ml of each) were diluted 10 times aiming to reduce the viscous nature of the extracts. The absorbence were then read on the spectrophotometer and their concentrations were determined from the standard curve. The values obtained were worked to original concentrations by considering the dilution factor of the extracts.

Microbiological quality of the extracts

This investigation was done to determine the microbial quality of the extracts in terms of total count, coliform and yeast and mould.

Enumeration of total count was done as described by IDF standard 100B:1991 while coliforms was done according to IDF standard 73A: 1985. Enumeration of yeast and moulds was done according to IDF Standard 94 B: 1990.

Standard plate counts, Coliforms and Yeast/mould were determined using plate count agar, red violet agar and potato dextrose agar (the medium was acidified to pH 3.5 with 10% sterile lactic acid) respectively. Serial dilutions of 1 ml of extract/10 ml quarter strength Ringer's solution was up to 10^{-3}.

Determination of chymosin and pepsin content in the extract

Determination of chymosin and bovine pepsin content in the three extracts and STD (25/75 chymosin and bovine pepsin) was done according to the IDF Standard 110A: 1987.

Preparation of the microgranulated DEAE cellulose and the column for determination of chymosin and pepsin was done as described by IDF standard 110A: 1987. Triplicate samples of extract (five ml each) and 1 ml of STD were used to determine concentration of chymosin and pepsin.

Effect of storage temperatures and containers

Effect of storage temperatures and containers on the shelf-life of the two extracts and STD were determined by using three different temperature regimes. Duplicate samples were stored at room (28-30 °C), refrigeration (5 °C) and freezing temperature (-18 to –20 °C) in glass and plastic containers and tested the MCA at 0, 2, 4, 8, 12, 16 weeks. Those stored in freezers were put in several containers according to the times of MCA determination to avoid re-thawing of the samples.

Determination of temperature and pH sensitivity of the extracts

Pepsin extracts are known to be very sensitive to temperature and pH changes, this study was undertaken to determine its sensitivity in milk.

For temperature sensitivity, reconstituted dried skim milk powder of pH 6.7 was set in duplicated samples at the following temperatures: 26, 28, 30, 32, 36, 38, 40, and 42 °C, in separate thermostatically controlled water baths and MCA was determined at the same milk pH of 6.7.

The pH was determined by adjusting the pH of the reconstituted skim milk in separate duplicate batches of pH 5.0, 5.5, 6.0, 6.5, 6.6, 6.7, 6.8, 6.9 and 7.0 with the addition of sour milk (pH 4.2) prepared from skim milk powder until the appropriate pH was reached. MCA was determined at the same temperature of 30 °C.

Comparison of formagraph coagulation characteristics of extracts

Duplicate samples of five ml of extract each and STD were standardised to the same clotting time of 7 min by diluting them with piperazine buffer pH 5.3.

Formagraph milk coagulation characteristics were determined as described in the Formagraph instruction manual using Formagraph equipment (Foss and Co. A/s, Hellerupvej, Denmark). When the coagulation was occurring, the resultant increase in viscosity and formation of the curd caused by the synchronous motion of the pendulum mirrors were recorded at different lateral positions on the chart and measured

Determination of proteolytic activity by agar diffusion diameter (AD) and by degradation of αs_1 casein

Proteolytic activity of the extracts were compared on their ability to hydrolyse the casein protein in milk. The agar gel plates preparation and determination of proteolytic activity by agar diffusion diameter was done according to Cheeseman (1963).

Proteolytic activity on αs_1 was done as described by Fox (1971). Separation of peptides by Reverse-phase Fast Protein Liquid chromatography (RP-FPLC) on Pep-RPC C_8 column and FPLC equipment (Pharmacia, 1993). TCA filtrate was diluted with buffer A (0.1% trifloroacetic acid -TFA, v/v and eluted using gradient of 80% acetonitrile analysis. The gradient was programmed and peaks were automatically printed and read comparing them with the standard peaks of the un-incubated sample.

3.1.5 The effect of Concentration methods on MCA of the extract

Three portions of 100 ml of each extract together with 1 g soluble starch were mixed and dried using three methods: freeze-drier, incubator and under direct sunshine respectively in duplicate samples to constant weights. Weights of the dried extracts were weighed and 1 g of each was reconstituted to make up for the moisture lost during drying. Clotting time was reported in min.

3.2 Experiment 2: Purification of bovine pepsin

3.2.1 Introduction

Purification is the process of getting rid of most or all the unwanted proteins and other substances from the crude extract and retain the desired enzyme of the highest possible purity with maximum possible yield and specific activity relative to the initial extract (Price and Stevens, 1991). Enzymes found in nature are in complex mixtures usually in cells, which perhaps contain several hundred different enzyme proteins. In order to study a given enzyme properly, it must be purified (Whitaker, 1972; Price and Stevens, 1991; Askura *et al.*1997). When a specific assay is used to study enzymes while they are still in impure state, in many cases some of the other enzymes present will interfere, either by attacking the substrate so as to give a side reaction or transforming the product into some other substances or by attacking the co-enzyme or even the enzyme itself. It is therefore sometimes difficult until the enzyme in question has been purified to say exactly what reaction it catalyses (Whitaker, 1972; Fox, 1987). Metabolic principles which have remained obscure and controversial have been put forward only after isolation of enzymes concerned (Fox, 1987). For studies of amino acid composition, molecular weight, primary,

secondary and tertiary structure, substrate specificity or kinetic parameters of a specific enzyme, it is necessary to completely separate the enzyme from other substances (Whitaker, 1972; Foltmann, 1987 and Fox, 1991).

Today, enzymes can be modified by genetic engineering techniques provided that they are first purified to homogeneity and their amino acid sequences determined (Farmakalids, 1995; Zalazar *et al.*1995 and Dajnowiec *et al.*1997). Concerning rennet preparations, cellulose in the coagulant, shortens the shelf life of their enzymes (Green, 1972). The proportion of the different enzymes in extracts may vary between batches. Therefore standardization of the milk clotting activity of the enzyme preparations becomes difficult (Garnot *et al.* 1972; Green, 1977 and Price and Stevens, 1991). This can affect the uniformity of the final products if the enzyme is not purified to the same level.

Therefore the aim of this experiment was to describe the purification steps of the extract obtained from experiment one, quantify and characterize the purified enzyme. Since purification and characterization procedures are expensive and time consuming, only one extract (HP) and STD were purified and detailed description of purification steps, quantification and characterization of HP extract will be shown.

Purification of HP was done at the Department of Food Science, Agricultural University of Norway. Both STD and HP extracts were purified through first step of purification in order to identify elution volume for chymosin and pepsin and then further purification of HP continued to the end.

3.2.2 Preparation of sample for purification

One hundred ml of HP (only 20 ml of STD was used) was concentrated to 50% of its original volume by ultrafiltration (UF) under 3.8 Atm using Amicon ultrafiltration cell (Amicon Model 52 from Amicon Corporation Lexington, Mass 02173 Holland) capacity 100 ml and Amicon UF membrane type 5 mm 05 43 mm lot AB 0697 with MW cut-off of 10 kDA. The concentrated 50 ml extract and 10 ml of standard STD (AB Chemicals, Mehno, Sweeden) were dialysed over-night against elution buffer A which was of pH 5.3 piperazine buffer 0.025 M.

The extracts were centrifuged in a Beckman CRR centrifuge (Beckman, UK) at 6,000 gavity (*g*) at 5°C for 25 min to make them ready for step 1 of purification by anion exchange chromatography.

3.2.3 Anion exchanger chromatography with DEAE Cellulose (step1)

The anion exchange chromatography column size 2.1 x 20 cm of DEAE cellulose was connected to the Biorad Chromatographic System Econo Rad (Biorad, Richmond, USA) equipment with a pump, gradient mixer, UV monitor operating at 280 nm and fraction collector.

The 25 ml of dialysed STD and 40 ml of UF concentrated, dialysed HP extract were applied on the column in separate runs and the proteins were eluted with the linear gradient of NaCl which was formed by mixing buffer A (0.025 M piperazine buffer pH 5.3) with buffer B (A + 0.7 M of NaCl). 140 fractions of 4 ml each (100 drops) were collected on a BIO-RAD fraction collector.

After completing the first step, the fractions were measured as follows:

1. Protein concentration was determined in the pooled fractions by reading the optical density (OD) at 280 nm in a Shimadzu spectrophotometer (Shimadzu, Tokyo, Japan).
2. The NaCl concentration in selected fractions was determined by titration with 0.1 $AgNO_3$ using potassium permanganate as an indicator, the end point was reached when the solutions became brick- red in colour.
3. Fractions were analysed for MCA at the pH of milk substrate of 6.0 and pH 6.4.
4. Proteolytic activity by agar diffusion (AD) method as described by Cheeseman (1963) was done to fractions which had high MCA.
5. Separated chymosin (eluted in the first peak) and pepsin fractions which had the highest MCA, OD (280 nm) and AD, were pooled, concentrated, dialysed and centrifuged before the next purification step.

3.2.4 Purification and determination of molecular mass by gel filtration (STEP II)

Gel filtration column containing ultrogel ACA capable of separating protein of molecular mass (MM) ranging from 25- 350 kDa were used. Protein Standards used for constructing a standard curve were

A. Catalase MM 232,000

B. Aldolase MM 158,000

C. Bovine serum albumin MM 66,000

D. Egg serum albumin MM 45,000

E. Trypsin inhibitor MM 20,000

The column was connected to the Biorad chromatographic equipment. Fractions corresponding to pepsin or chymosin peaks for STD and HP from step 1 were separately concentrated to 6.5 ml by UF as described above. The concentration was necessary because a small volume of sample (below 5% of the total gel bed volume) was necessary to assure good separation by gel filtration chromatography.

The Pharmacia LKB Biotechnology (Uppsala, Sweeden) K 21 column was filled with ultrogel ACA (Pharmacia LKB Biotechnology) to form a 120 cm high and 2.1 cm diameter bed. The column was equilibrated with buffer A containing 0.15 M NaCl, the NaCl was added to reduce unspecified absorption. As measured with blue dextran and potassium chromate, the void and the total volume of the column were 72 and 450 ml, respectively. A mixture of protein standards were run first on the column in order to construct a standard curve for determination of molecular mass. About 5.4 ml out of the 6.5 ml concentrated pepsin, was loaded on the column. The flow rate was 1 ml per min and fraction size was 4 ml. Ninety eight fractions were collected for OD, MCA and AD were determined and the most active fractions were pooled for the third step of purification. Step II was repeated for chymosin purification after equilibration of the column.

3.2.5 Chromatography on Mono Q anion exchange

Step 111

The pepsin fraction from the step II was chromatographed on high performance anion exchange monodisperse bead column (Mono Q HRE/5/5 5mm x 5 cm Pharmacia LKB Biotechnology). The column was fitted to Fast Protein Liquid Chromatographic (FPLC) equipment (Pharmacia). The equipment contained 2 pumps

(gradient maker), electronic programmable unit, monitor, two-pen recorder to monitor absorbency and gradient, programmable multi-position valves for sample loading and injection and super loop to inject samples of volume 1-50 µl.

The column was equilibrated with piperazine buffer (buffer A) and proteins were separated using NaCl gradient after putting on the column, 12 ml sample from step II of purification. The gradient was programmed on the FPLC microprocessor unit to elute the pepsin through the column using buffer A and buffer B, the same as used in the first step of purification. The flow rate was 0.5 ml/min. The sample before application to the column was equilibrated with buffer A by dialysis overnight at 4°C and centrifuged at 7000 g for 10 min. Thirty six fractions of 1.5 ml each were collected. OD, MCA and AD were determined as in step 1. The most active fractions were pooled and dialysed against buffer A to be used for step IV. Step 111 was repeated for chymosin purification.

3.2.6 Phenyl Sepharose on FPLC (Step 1V)

Purification by Phenyl Sepharose works on the principle of hydrophobic interaction. Phenyl Sepharose column type HR5/10 size 5 mm x 10 cm (Pharmacia) was fitted to FPLC equipment. Following instructions given by the column manufacturer, the linear gradient of 1.7 M ammonium sulphate in the piperazine buffer (buffer A) down to zero concentration of ammonium sulphate. Buffer A was used to equilibrate the sample collected from Mono Q by dialysis. The flow rate was 0.5 ml/min. 40 fractions of 1.2 ml each were collected and the analysis for the fractions were done as in step 1 which yielded 3 partially separated peaks (named A, B and C, Figure 16)

3.2.7 Rechromatography of peak A on Mono Q

The fraction corresponding to the first peak (A) of protein and which was the most active, (50 µl) was rechromatographed on Mono Q equilibrated column which yielded a symmetrical and superimposed sharp peak of MCA and proteolytic activity. From each pooled fraction of each step of purification, small samples were collected for analysis of MCA and protein content in order to calculate recovery and purification efficiency. Small sub-samples taken for SDS-PAGE were additionally dialysed against piperazine buffer diluted 1:1000.

3.2. 8 Characterization of purified enzyme

Determination of optical density (OD) at 280 nm

One ml of the extract was diluted with 5 ml acetate buffer pH 5.5 and then the OD at 280 nm was measured in spectrophotometer. The increase of one unit in absorbence at 280 nm in a cuvette with 1 cm light path corresponds to protein content 1µl /ml.

Determination of MCA

The MCA of the purified fractions was done as described 3.1.2 (iii)

Determination of proteolytic activity by agar diffusion diameter (AD)

Proteolytic activity determination by AD was done as described earlier (3.1.4) fractions which were most active in MCA during different steps of purification were used for this determination.

Determination of molecular mass (MM)

Buffer : B= A diluted 4 times containing 0.15 M NaCl.

Protein standards (SIGMA) for constructing the standard curve.

Egg albumin	MM 45,000
Bovine serum albumin	MM 66,000
Aldolase	MM 158,000
Catalase	MM 232,000
Blue dextran	MM 2,000,000

Protein standards were weighed (5 mg each) and dissolved in 6 ml of distilled water. The mixture was eluted on gel filtration column with buffer B. One hundred and fourty three fractions (each 4 ml) were collected and their OD were determined. An OD graph was plotted against the fraction numbers to show the peaks of the protein standards.

The void (Vo) volume (the eluted volume of blue dextran) and the volume of eluted protein standards (Ve) were recorded. The linear relationship was obtained when the logarithm (log) of molecular mass of the protein standards were plotted against respective log ratio (Vo/Ve).

Electrophoresis in polyacrylamide gel with sodium dodecyl sulphate (SDS-PAGE)

Sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) was performed using Pharmacia Phast-System with ready cast 12% gels and SDS saturated electrode strips. The procedure is based on the original method of Laemmli

(1970). The extract and pooled samples of pepsin from all four steps of purification, each 0.2 ml, were dialysed against buffer A. They were transferred into eppendorf tubes after washing the dialysing tubes well to ensure removal of all the sample. A small hole was made on the cover of the eppendorf tubes and covered further with aluminium foil after labelling the tubes.

The samples were concentrated by freeze drying. The samples were dissolved with 25 µl of SDS sampling buffer and heated in boiling water for 1 min. One ml of each mixture was applied on the gel in order to estimate the molecular mass (MM). In order to increase sensitivity, the gels were stained using Colloidal Brilliant Blue G (SIGMA), the proteins were fixed and the gels destained according to the procedure given by the manufacturers.

Fixing solution was 7% acetic acid in 40% methanol

Destaining solution was 30% methanol in 10% acetic acid

Preserving solution was 5% gycerol in 10% acetic acid

Amino acid sequence

Purified pepsin from step III and IV were freeze dried and sent to Biotechnology Centre, University of Oslo for N-terminal amino acid sequence determination.

Purified pepsin was sequenced by Edman degradation on an automated pulsed liquid-phase protein-peptide sequencer (Applied Biosystems Inc. Foster City, CA, USA; model 477A). Liberated amino acids were detected as their phenylthiohydantoin derivatives using a model 120 Analyser (Applied Biosystems). Swiss-Pro. data base was used for comparison of N-terminal sequencies.

Measurement of formagraph coagulation characteristics for fractions pooled through purification procedures

Formagraph coagulation characteristics were analysed as was described by McMahon and Brown (1982) and as described by N. Foss and Co A/S. Pepsin purified in steps I, II, III and STD, all standardised to the same clotting time (7 min) with piperazine buffer pH 5.3. and characterised for their coagulation ability.

3.3 Experiment 3: Assessment of cheesemaking properties HP and VP extracts

3.3.1 Introduction

The ultimate method for assessment of the suitability of a coagulant is using it in cheese manufacture (Fox, 1987, 1993). Commercial rennet (CR) is usually used as a control in cheese trials because of its chymosin content which results in a more specific milk coagulating activity than with pepsin or other enzyme alone (Medina *et al.*1992). However, time and cost make it desirable to carry out a simple screening procedure even before small scale cheese making is undertaken (Green, 1977). Cheese manufacture is essentially a dehydration process in which casein, fat and minerals are concentrated 6 - 12 fold (Fox, 1993; Hill, 1995). It varies greatly between many types of cheese produced.

According to the conclusions made in experiment 1, the abomasa extracts with HCL (HP) and vinegar (VP) respectively, were chosen for assessment in cheese production experiments. The extract with lemon (LP) was too proteolytic, had a short shelf life, contained less rennet activity and required more clarifying reagent.

The aim of this study was to further investigate the suitability of HP and VP for the cheese manufacture. The extracts were combined with commercial rennet at different levels. The cheese samples withdrawn from the cheese at different intervals were analysed chemically and microbiologically. Sensoric evaluation was carried out after the cheese had matured, and the overall quality assessments of cheese characteristics were compared to determine the best enzyme combination for each cheese type.

Comparison between different enzyme combinations were made within each type of cheese according to the location where it was made and not between cheese types.

Main objectives:

1. Determine the renneting times of the different CR/pepsin combinations used to coagulate SUA Alpine and SUA Pasta cheese milk and loss of TN and BF in whey during cheesemaking.
2. Investigate the changes in chemical composition of cheese made with different CR/pepsin combinations during cheese ripening.
3. Determine the microbial quality of cheese made with different enzyme combinations during cheese ripening
4. Determine sensory characteristics of the cheese made with different enzyme combinations at maturity.
5. Determine the best level of CR/pepsin combinations suitable for each type of cheese.

3.3.2 Methodology

Preparation of different combinations of CR/pepsin for cheesemaking

Duplicate samples of HP, VP extracts and CR were analysed for their milk clotting activities according to IDF Standard 110 1987. HP and VP extracts were then mixed with CR at different ratios and amounts were measured according to their pepsin units (PU) (Table 4) and stored in bottles ready for use in cheesemaking

Table 4: Percentages and amounts of CR and pepsin extracts (ml) combinations for cheesemaking

Level of HP or VP	Enzyme Type	% HP or VP	% CR	Amounts HP or VP	Amounts CR (g)
100CR	CR	0	100	0.0	1.2
25HP	HP	25	75	16.	0.9
50HP	HP	50	50	32.	0.6
75HP	HP	75	25	48	0.3
100HP	HP	100	0	64	0.0
25VP	VP	25	75	6	0.9
50VP	VP	50	50	13	0.6
75VP	VP	75	25	19	0.3
100VP	VP	100	00	25	0.0

These enzyme combinations were used to coagulate 40 l of milk

All levels of CR/pepsin were used in making each type of cheese and the 1st level (100CR) was used as the commercial rennet. The coding given in the first column of

Table 4 showing the level of pepsin was used throughout the text to represent the enzyme combination and the name of cheese.

Cheese made at SUA Laboratory conditions

SUA Alpine and SUA Pasta types of cheese were made in the Dairy Technology Laboratory of the Department of Animal Science and Production at Sokoine University of Agriculture (SUA), Morogoro where the manufacturing conditions for the cheese were suitably controlled. The cheeses were thus named SUA Alpine and SUA Pasta. Milk was obtained from Magadu research farm belonging to the same department. Commercial rennet of 98% chymosin and 2% pepsin of 1000 International milk clotting unit (IMCU)/g was obtained from Chr. Hansen's Laboratory. Denmark.

Cheese made under field conditions

Alpine and Pasta cheeses were also made by two small scale cheese processors (SSCP) who normally make these types of cheese at Nkoaranga Village on the slopes of Mount Meru near Arusha. This was done to investigate the effect of differences in manufacture and storage conditions for the different enzyme combinations in the production of these types of cheese. All these cheeses were coined Village. In addition Tilsiter cheese was also made under Village conditions to investigate if the type of milk treatment for cheesemaking would show any differences with the enzyme combinations. Usually Tilsiter cheese is made from half pasteurised milk and half raw milk. The SSCP obtained milk from their farms and from their neighbours who keep dairy cows.

3.3.3 Cheesemaking

Pasta and Alpine cheese both at SUA and Village conditions and Tilsiter cheeses were made according to the procedure described by FAO-RDDTT (1990), see Appendix (1.5-1.7).

For the SUA cheese, duplicate cheeses of two kg each were made from each combination each day. Normally Alpine and Tilist mature for 42 days while Pasta mature for 14-21 days. Therefore the main discussion on cheese was centered on 42 and 14 days respectively. In order to see the effect of overipening the cheese, Alpine and Tilist were over-matured up to 56 days while Pasta for 28 days. Samples were taken from SUA Alpine after 2, 21, 42 and 56 days while for SUA Pasta at after 2, 14 and 28 days. For Village cheeses it was difficult to drill samples aseptically and store them. Eight cheeses of one kg each were made for each combinations per day. Two cheeses were labelled according to the days of sampling and on the day of sampling the cheeses were thawed for sample taking at SUA.

3.3.4 Determination of renneting time (RT) of the enzyme combinations and TN and BF losses in whey

RT, TN and % BF were determined only for cheeses made at SUA because of availability of equipment. RT determinations were done for each enzyme combination for both types of cheese to measure time taken from enzyme application until curd was firm enough for cutting. This was done twice according to FAO- RDDTT (1990).

Dublicate samples of whey for determination of TN and % BF for each enzyme combination were taken and analysed.

3.3.5 Cheese sample preparations

Cheese sample preparation was done according to IDF Standard 4A 1982

Prior to sample drilling, aseptic conditions were observed, the rind or smear or mouldy surface layer of cheese was removed to provide a representative sample. The samples were wrapped in aluminium foil, placed in air tight container and frozen at -20^0 C until time for analysis when the samples were grated immediately, the mass was mixed and chemically analysed as soon as possible.

3.3.6 Cheese quality assessment

Cheese evaluation was carried out using three major criteria namely chemical composition; microbial quality and sensory evaluation. Parameters measured in each criterium are described below:

The major characteristics measured in this criterium are:

i) Chemical composition of cheeses

The pH and acid in cheese

The pH and acid were determined in cheese as described by Egan *et al.* (1981).

Moisture content

Moisture content determination was done according to Egan *et al.* (1981). Duplicate cheese samples of three grams from each enzyme combination were weighed and dried in the oven at a temperature of 102°C for 3 hours.

Fat content

Fat content was determined according to Egan *et al.* (1981)

NaCl content:

NaCl was determined according to Association of Official Analytical chemists (1975) Duplicate cheese samples each weighing 2 g from each enzyme combination were put into a 200-500 ml conical flasks and 25 ml of 0.05 M silver nitrate solution and 10 ml water was added to each. Dispersing of the cheese was by swirling and warming to 80°C while adding 10 ml concentrated nitric acid to each sample and boiling gently for about 10 min until the precipitate was granular. Then 0.5 g of urea was added to the hot solution, cooled and 1 ml nitrobenzene and 50 ml water was titrated with the un reacted silver nitrate was titrated with 0.01 M potassium thiocyanate solution using ferric alum as indicator. Calculations were done based on the assumption that: 1 ml 0.05 M $AgNO_3$ ≡ 0.002922 g NaCl.

Formula for calculating NaCl content was as follows:

% NaCl = ml potassium thiocynate solution (minus blank) x 0.5844 (normality of NaCl divide by 2gm (sample)

Total nitrogen (TN)

Total nitrogen was determined by the Kjeldahl method according to AOAC 1969 Standard 15.017 in duplicates for each enzyme combination with some modifications as recommended by the manufacturers of the Tecator Kjeltec 1002 Distillation unit. Chemical analysis was carried out for 0.5 g of each cheese sample.

Water soluble nitrogen (WSN)

Water soluble nitrogen was analysed according to Ling (1963). Duplicate cheese samples of 2 gm each from each enzyme combination was transferred into a mortar and 25 ml of warm distilled water (40°C) was added to each sample. The samples were ground, and the solubles were transferred into measuring cylinders by decanting. The remaining residue in the mortar was macerated, warm water added and the solubles were again decanted into the same measuring cylinders. This was repeated until a volume of 100 ml was obtained for each sample. The extracts were filtered. Five ml (representing 0.1 g of cheese) of the filtrate was digested as described under total nitrogen determination. The results of TN, BF, NaCl and % WSN were presented on dry matter basis (DM)

Cheese ripening index

Ripening index as a measure of ripening or proteolysis was calculated by dividing water soluble nitrogen (WSN) by total nitrogen (TN) and multiplied by 100.

ii) Cheese microbiological quality

Microbiological quality of the cheeses was analysed as described above (3.1.4 (d) of experiment 1). The potato dextrose agar was acidified to pH 3.5 by 1N tartaric acid and serial dilutions of 1 g of each cheese per10 ml peptone water were made up to 10^{-6} and one ml of 10^{-4}, 10^{-5}, and 10^{-6} were used for cheese microbial determination. Standard plate count, coliform and yeasts and mould were determined in cheese because these and others may lead to physical and chemical defects during ripening and cause alteration in texture, flavour, body and appearance of cheese. In order to differentiate

the defects due to enzyme combination and effects due to microbial quality, microbial enumeration was necessary.

iii) Sensory evaluation of cheese

Semi-trained panellists (15-20) mainly consisting of workers from the Department of Animal Science and Production and Food Science and Technology at SUA formed a panel of judges for the ranking test. The panellists were women and men of middle age. Cheese samples were prepared by peeling off the rind and slicing cheese into pieces of equal size and wrapped in aluminium foil.

Description of panellists task and presentation of the samples:

Panellists were each presented with nine well coded samples in identical sample containers coded with 3-digit random numbers. All the samples were simultaneously presented to each panellist in a balanced or random order. They were asked to rank coded samples for intensity of taste and smell, appearance and bitterness by ordering the samples from 3 (being very good), 2 (for good) and 1 (for poor) in taste, smell and appearance. Ranking for bitterness was 3 (for not bitter), 2 (for slight bitter) and 1 (for very bitter). They were required to taste the samples in the order presented to them and check how much they liked or disliked each sample. They were allowed to re-evaluate the samples as necessary to make the required comparison among them. Thereafter, the panellists were required to give a comment. A ballot used for ranking intensity of the cheese has been attached as Appendix (1.8). There were separate sessions in which ranking tests were carried out on separate days. When all the panellists had ranked the samples, the ranks assigned to each sample were totalled. The samples were then tested

for significance of differences by the Benfferoni test to give differences between enzyme combination and hence suitable for each type of cheese.

3.3.7 Statistical analysis

A complete randomised design was used in analysis of the cheese data obtained. This was performed on the SAS (1992) (proc GLM) package, for enzyme combination and cheese age as main effects. Analysis of Variance, (ANOVA) was performed to establish any significant differences among the level of CR/pepsin used and storage for the type of cheese. ANOVA Tables, for the chemical composition of cheeses up to overripening time were given in Appendix 1.9 while weekly Least Square means (LSmeans) and standard error of chemical composition and microbial quality of cheese up 28 and 56 days have been presented in the text in a form of graphs. LSmeans and standard error of chemical composition and microbial quality of cheese at maturity have been presented in the text in a form of Tables.

The statistical model was:

$$Y_{ijkl} = \mu + L_i + A_j + (LxA)_{ij} + E_{ijkl}$$

where:

Y_{ijkl} = Effect of quality of cheese

μ = the overall mean

L_l = Effect of level on pepsin in cheese

A_j = Effect of age on pepsin in cheese

$(LxA)_{ij}$ = Effect of level pf pepsin and age of cheese

E_{ijkl} = Residual effect

Sensory evaluation for taste, smell, appearance and bitterness was analyzed by one way analysis of variance with Bonferroni test to find the differences between the enzyme combinations within the type of cheese. The ANOVA Table for sensory evaluation were given in Appendix 1.10 while the mean squares of the sensory characteristics and their standard error were presented in the text in form of Tables Overall cheese assessment of quality for chemical composition, microbial quality and sensory evaluation, arbitrary grades were made from ranges found in literature or were compared with their respective controls and then there were analysed by Bonfferoni test to give differences between enzyme combination suitable for each type of cheese. The summary of cheese grade means and their standard error have been presented in the Appendix 1.11-1.15.

The data for ranking were analysed by ANOVA to establish any significant differences among the enzyme combinations in cheeses.

The statistical model was:

$$Y_{ij} = \mu + X_i + E_{ij}$$

where: μ = the overall mean

X_i = Effect of pepsin treatment

E_{ij} = Residual effect

CHAPTER 4

RESULTS

4.1 Experiment 1: Extraction of bovine pepsin

4.1.1 Optimisation of extraction conditions for maximum MCA of crude extract

Fundic versus pyloric regions of abomasa

The pepsin concentration in the fundic and pyloric regions of the abomasa (Table 5) were significantly different ($P< 0.001$). The protein content was 1.076 mg/ml and 0.459 mg/ml and the average content of pepsin were 70.2 % and 27.4 % of the total milk clotting enzyme in the fundic and pyloric regions, respectively.

Table 5: Protein concentration (mg/ml) and percentage of pepsin in the fundic and pyloric region of the abomasa

Amount pepsin	N[1]	Protein mg/ml LSmeans (±se)	% pepsin	Pr > T
Fundic	14	1.076 (0.053)[a]	72.2	0.001***
Pyloric	14	0.459 (0.053)[b]	27.4	

[1] Number of observations

*** Highly significant different

Within the column means followed by the same letter do not differ significantly (P>0.05) according to Duncan multiple range test (DMRT)

Dried versus fresh abomasa

Results (Table 6) showed that dry abomasa gave an extract with significantly ($P< 0.05$) higher MCA than fresh abomasa. Since dried fundic region of the abomasa gave better results, it was used for pepsin extraction in all subsequent pepsin extraction experiments.

Table 6: Milk clotting activity (PU/ml) from extracting fresh versus sun dried abomasa

State abomasa	N	Protein mg/ml Lsmeans (±se)	Pr > T
Dry	14	3296 (110)[a]	0.001***
Fresh	14	2326 (110)[b]	

Within the column means followed by the same letter do not differ significantly (P>0.05) according to DMRT.

Effect of drying methods

Drying methods (Table 7) differed significantly ($P<0.05$) among the various parameters studied. Sun drying gave (1987 PU/ml) extracts with more MCA than shade-drying (1800 PU/ml) and oven drying (1609 PU/ml). The duration of drying fundic region of abomasa to constant weight was 4 days under direct sunlight, 6 days under shade and 7 days in the oven although the drying temperatures were nearly the same.

The percent dry matter content of the three extracts differed significantly ($P< 0.05$). Calculated means were 15.73 and 16.73 % for sun dried and shade dried respectively.

The microbial quality showed no significant difference ($P>0.05$). The calculated mean values of 2.3×10^4 and 2.7×10^4 cfu/g were obtained in extracts from sun and shade dried preparations, respectively. In subsequent experiments, pepsin extraction from the fundic region of the abomasa were from sun dried preparations.

Table 7: Effect of drying cattle abomasa under direct sunlight and under the shade on the MCA (PU/ml), dry matter and bacterial count (cfu/ml)

Drying methods	N	Parameters determined (±se)			Pr>F
		Pepsin PU/ml	%Dry matter	SPC Cfu/ml	
Sundrying (25-30^{0}C)	6	1987 ± 19^a	15.73 ± 19^a	$2.3 \times 10^4 \pm 19^a$	0.009***
Shade (25-30^{0}c)	6	1800 ± 14^b	17.30 ± 14^b	$2.7 \times 10^4 \pm 14^a$	0.001 ***
Oven (30^{0}c)	6	1609 ± 33^c	ND	ND	0.033 **

** Significant difference ND - Not done

*** Highly significant difference

Within the column means followed by the same subscript do not differ significantly (P>0.05) according to DMRT.

Optimum NaCl concentration of the extract

ANOVA Table 1 in Appendix 1.1 shows that MCA of extracts with different ionic strength were significantly different ($P<0.01$) between days and highly significantly different ($P<0.001$) between levels of NaCl. The highest MCA of the extract was obtained at 10 % NaCl concentration (Figure 6). Above this there was an inverse relationship between NaCl concentration and MCA. In the subsequent experiments 10% NaCl was used in the pepsin extraction.

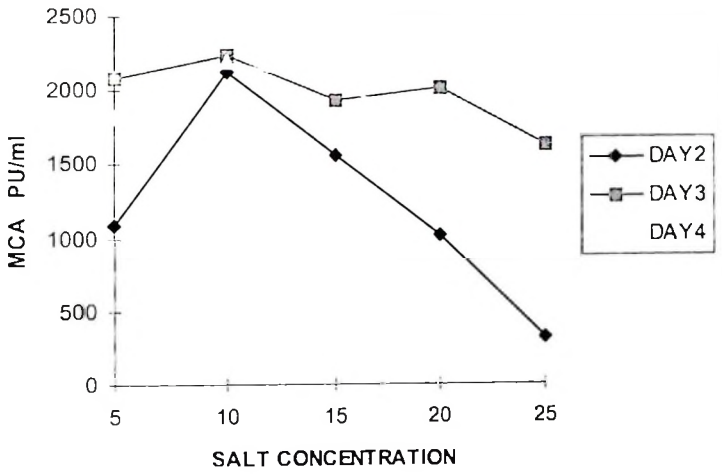


Figure 6: Effect of ionic strength and extraction period on milk clotting activity (MCA) PU/ml of the sun dried fundic region of the abomasa

Effect of Storage temperatures and type of container

Figure 9 shows that when HP was stored at room temperature in glass containers it was active up to 6 months. But after that it started losing activity, while the sample stored in plastic bottles lost activity within two and a half months. HP stored in the refrigerator and kept in glass container was fairly active for more than 6 months but the one kept in plastic container started losing activity at 2-3 months and more so at 5-6 months. HP stored in deepfreezer kept in glass and plastic containers had same activity for more than 6 months although the one in glass had higher activity than the one in plastic containers.

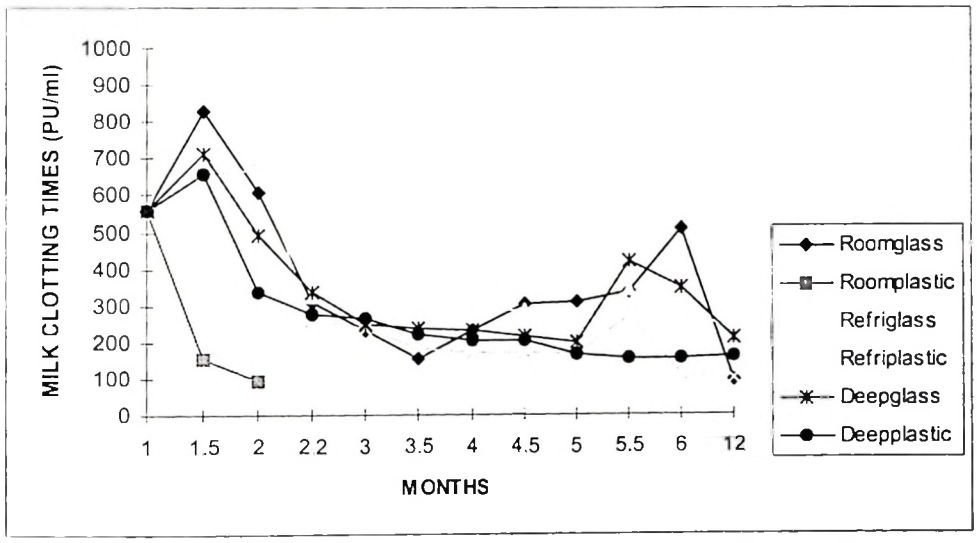


Figure 9: Effect of storage temperatures and type of container on MCA of HP extract

Figure 10 shows that the MCA of the VP extracts stored in both glass and plastic containers at room and refrigeration temperatures did not show much change in MCA after 6 months of storage but keeping VP extract frozen in plastic containers for four months lead to a reduction in MCA to zero.

Optimun extraction temperature

Extraction temperatures (Table 8) lead to a significant difference in MCA ($P<0.05$) between 37 °C (2727 PU/ml), room temperature (1889 PU/ml) and refrigeration temperature at 5 °C (332 PU/ml). In spite of the high MCA obtained at 37 °C, the extraction of pepsin was subsequently done at room temperature because there was a frequent electricity power failures causing difficulties during incubation period.

Table 8: Effect of extraction temperature on MCA of crude extracts

Temperature	N	37^0C Oven	25-30^0C Room	< 10^0C Refrigerator
PU/ml	6	2727^a	1889^b	332^c

Within the row means followed by the same letter do not differ significantly (P>0.05) according to DMRT.

Determination of optimum extraction pH and time

ANOVA Table 2 in Appendix 1.2 shows that the effects on MCA of crude extracts were significantly different ($P<0.001$) for both pH levels and time used for pepsin extraction. Maximum MCA was obtained when the dried abomasa was macerated at pH 3 for 3 days (Figure 7).

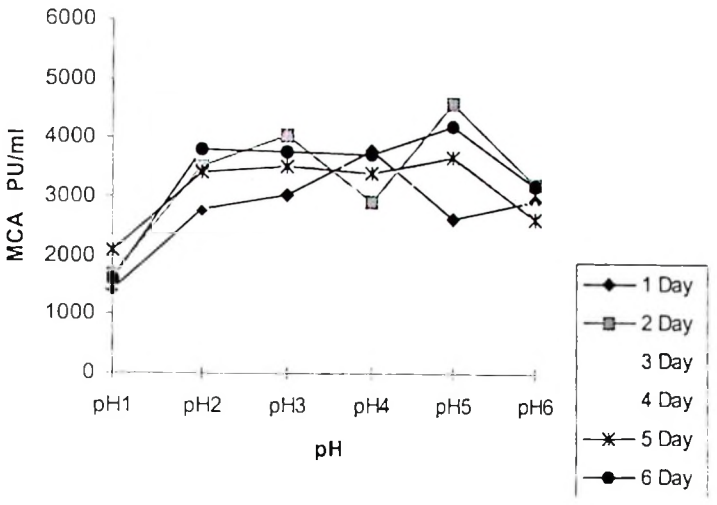


Figure 7: Effect of extraction time and pH on MCA crude extracts from the sun dried fundic region of abomasa

Effect of activation pH and time

MCA from activation at pH 1, 2, 3 and 4 were significantly different ($P<0.05$) (Figure 8). The curve of pH 1 reached maximum MCA at 40 min but it was unstable with time. At pH 2 the maximum MCA was at 30 min and the curve was stable up to 60 min showing that all the pepsinogen had been converted to pepsin. At pH 3 the curve was also unstable although the maximum MCA was reached at 20 min. pH 4 gave the lowest yield therefore pH 2 for 30 - 60 min was taken as the appropriate activation pH and time and was to be used in subsequent experiments.

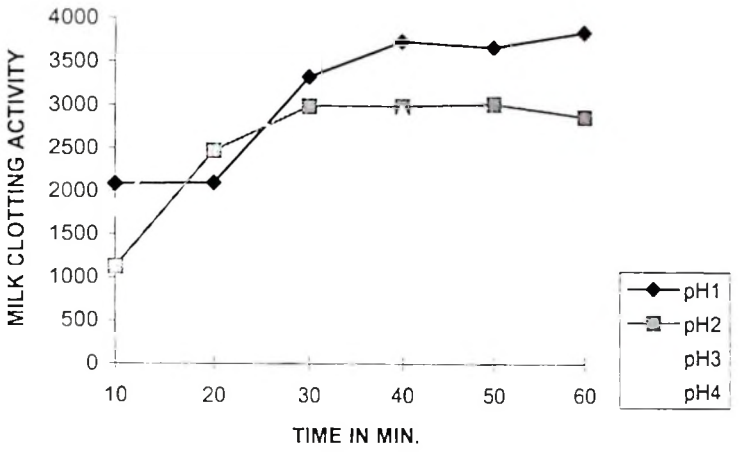


Figure 8: Effect of activation pH and time on MCA of the crude extracts of the sun dried fundic region of the abomasa

Effect of clarification methods

- Centrifugation speed

There was no significant difference ($P>0.05$) between centrifugation speeds used (Table 9a). Despite the lack of significant difference, by means of separation, there were observable differences where 3000 *g* for 15 min gave the highest MCA while 1000 *g* for 15 min gave the lowest MCA

Table 9a: Effect of centrifugation speed on MCA of extracts

g/15 min	N [1]	1000	2000	3000	4000
PU/ml	8	860 ±13[a]	889 ± 9[a]	902 ± 10[a]	899 ± 5[a]

[1] Number of observations

Within the row means of MCA/ml followed by the same letter do not differ significantly according toDuncan's multiple range test

- Gravitational sedimentation, centrifugation and filtration

When the MCA of the three methods of clarification were compared, (Table 9b), filtering gave preparations with longer clotting time followed by centrifugation and finally gravitational sedimentation. The increase in MCA was slightly higher for the gravitational sedmentation method.

Table 9b: Effect of physical clarification treatments on milk clotting time.

Treatment	N [1]	Milk clotting activity (min)	% increase of MCA
No treatment	20	2.00	0
Gravitational sedimentation	20	1min 13sec	39.2
Centrifugation	20	1 min 20 sec	33.3
Filtration	20	1min 25 sec	29.2

[1] Number of observations

- Clarifying with chemicals

Table 9c shows that the use of $Na_2HPO_4.H_2O$, Na_2HPO_4, Na_2HCO and NaOH for clarification, resulted in average clotting times of 2.58 min, 3.10 min, 3.45 min, and 4.30 min respectively. The former two chemicals did not perform significantly different ($P>0.05$) but were more efficient in separating the abomasal tissue from the enzyme than the latter two chemicals which also gave no significant difference ($P>0.05$) from each other. All of them were significantly different from the control preparation ($P<0.05$).

Table 9c: Effect of the clarifying chemicals on MCA.

Chemicals	N	Milk clotting time (min)
No treatment	12	6.00 min[c]
$Na_2HPO_4.H_2O$	12	2 min 58sec[a]
Na_2HPO_4	12	3.min 10sec[a]
Na_2CO_3	12	3 min 45sec[b]
NaOH	12	4min 30sec[b]

Within the column means of PU/ml followed by the same letter do not differ significantly according to DMRT

Effect of different storage temperature regimes

Table 10 shows that there was no significant difference ($P>0.05$) between MCA of extracts stored at ambient temperature and those at refrigeration temperature while MCA of the extract stored in the deep freezer and the freshly prepared extract differed significantly ($P<0.001$) from the other two treatments.

Table 10: Effect on MCA of different storage temperature regimes of the clarified extracts for 6 weeks

Storage temperature	N	Pepsin units/ml (LSmeans)	Standard error	Pr > F
Initial	6	562[c]	97	0.001
Room	6	1049[b]	97	0.05
Fridge	6	1030[b]	97	0.05
Freezer	6	1376[a]	97	0.01

Within the rows means of PU/ml followed by the same letter do not differ significantly according to Duncan's multiple range test

Effect of interaction of extraction conditions

Interaction of extraction conditions (Appendix 1.4) shows that interaction between temperature x NaCl and days of extraction x NaCl were not significant ($P>0.05$). However, interaction between days of extraction x temperature and days of extraction x pH were observed to cause significant effect on MCA of extracts ($P< 0.01$) while NaCl x pH and temperature x pH caused highly significant effect on MCA of extracts ($P< 0.001$).

4.1.2 Summary of optimum extraction conditions leading to maximum MCA of the crude pepsin extract using dilute HCl

After determining optimum extraction conditions leading to the maximum MCA, results showed the following:

1. The fundic region had the highest pepsin concentration (1.076 mg/g)
2. Dried fundic region of abomasa gave higher MCA than fresh ones
3. Sun drying was the best method of drying the fundic region of the abomasa
4. NaCl concentration of 10 % is the best ionic strength for crude pepsin extraction
5. Best pepsin extraction pH was 3 for 3 days
6. Best extraction temperature was 37 °C but since it needed an oven for incubation to maintain the temperature, room temperature was found to be more appropriate and cheap for small scale cheese processors
7. Activation pH and time of the crude pepsin was 2 and 30 min
8. Best clarification methods for crude pepsin extract were found to be:
   - centrifugation at 3000g for 15 min
   - chemical clarification was best with $Na_2HPO_4.H_2O$
9. Refrigeration and deep freezing were best storage temperatures when kept for more than 6 months.

4.1.3 Characteristics of the enzyme extracts

Using these extraction conditions which lead to maximum MCA, bovine pepsin was extracted using three different extraction solutions namely dilute HCl, Lemon juice and Vinegar. The pepsin extracts were coded HP, LP and VP extracts respectively. They were characterized and compared with the STD

The physical characteristics of the extracts

The colour, taste, odour and clarity of the extracts were compared to STD. HP and VP had a brownish yellow colour while LP was pale yellow. Taste for all the extracts was similar to STD. The smell of STD was like alcohol, HP had no smell while VP and LP smelt like vinegar and lemon, respectively. Both HP and STD were turbid while LP and VP were slightly turbid.

The pH and acidity

The crude filtered HP, LP, VP extracts after maceration for 3 days and STD had pH 4.47, 2.64, 3.42 and 5.85. Their acidities were 1.70, 0.22% citric acid, 12.5 % acetic acid and 2.4% respectively. LP had both low pH and low citric acid content.

Milk clotting time

MCA of STD, HP, LP and VP extracts were 0.52, 0.50, 1.3 and 0.60 min respectively, LP extract used more time to coagulate milk than others.

Total solids (TS) content of the extracts

TS content of the extracts were 27.9, 24.4, 23.5 and 16.4 % for LP, VP, STD and HP extracts respectively. LP extract had the highest TS content.

Total nitrogen of the extracts

Total protein content of the extracts were 1.2, 1.3 and 1.1% for HP, LP and VP extracts respectively.

Microbial quality of the extracts

The three extracts had no coliform bacteria and no yeast/mould but LP had less than 10 cfu/ aerobic count at 30 ^{0}C / ml of the extract just after clarification. The extracts were preserved with 0.2 % sodium benzoate.

Chymosin and pepsin contents

Table 11 shows that the STD extract which was labelled 25/75 (chymosin/pepsin), had almost the same chymosin/pepsin content as stated. The chymosin content of the three extracts by IDF Standard 110a: 1987 were significantly different ($P<0.05$) from each other. The HP contained more chymosin followed by VP and LP extract. So the extracts contained mainly pepsin and very little chymosin.

Table 11: Percent content of chymosin and pepsin isolated from the chemically clarified extracts

Extracts	N	Percentage chymosin and pepsin (±se)	
		Chymosin	Pepsin
HP	4	3.5 (0.16)^a	96.5 (0.16)^a
LP	4	0.9 (0.16)^b	99.1 (0.16)^b
VP	4	2.6 (0.16)^c	97.4 (0.16)^c
STD	4	24.5 (0.16)^d	75.5 (0.16)^d

Within column, means followed by the same letter do not differ significantly at (P>0.05) according to DMRT

Effect of Storage temperatures and type of container

Figure 9 shows that when HP was stored at room temperature in glass containers it was active up to 6 months. But after that it started losing activity, while the sample stored in plastic bottles lost activity within two and a half months. HP stored in the refrigerator and kept in glass container was fairly active for more than 6 months but the one kept in plastic container started losing activity at 2-3 months and more so at 5-6 months. HP stored in deepfreezer kept in glass and plastic containers had same activity for more than 6 months although the one in glass had higher activity than the one in plastic containers.

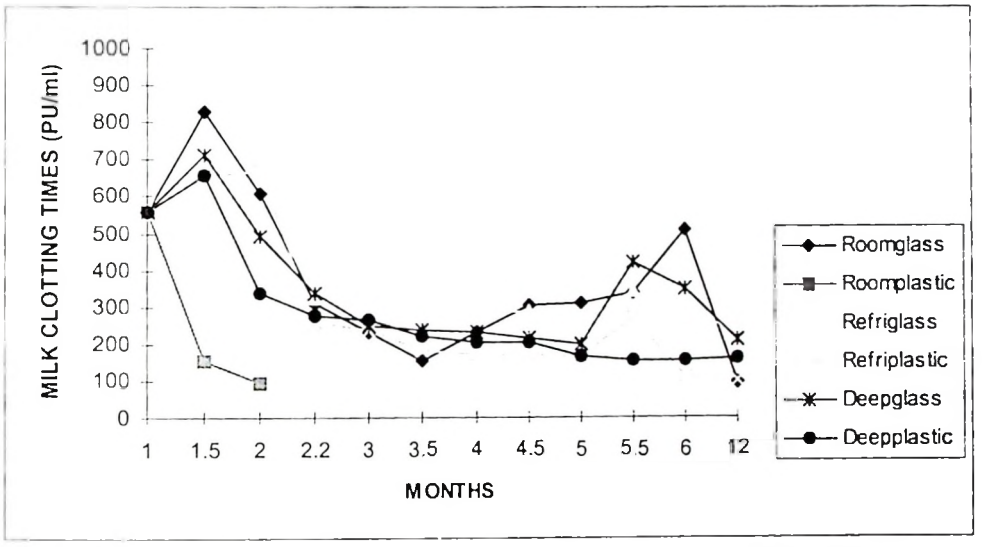


Figure 9: Effect of storage temperatures and type of container on MCA of HP extract

Figure 10 shows that the MCA of the VP extracts stored in both glass and plastic containers at room and refrigeration temperatures did not show much change in MCA after 6 months of storage but keeping VP extract frozen in plastic containers for four months lead to a reduction in MCA to zero.

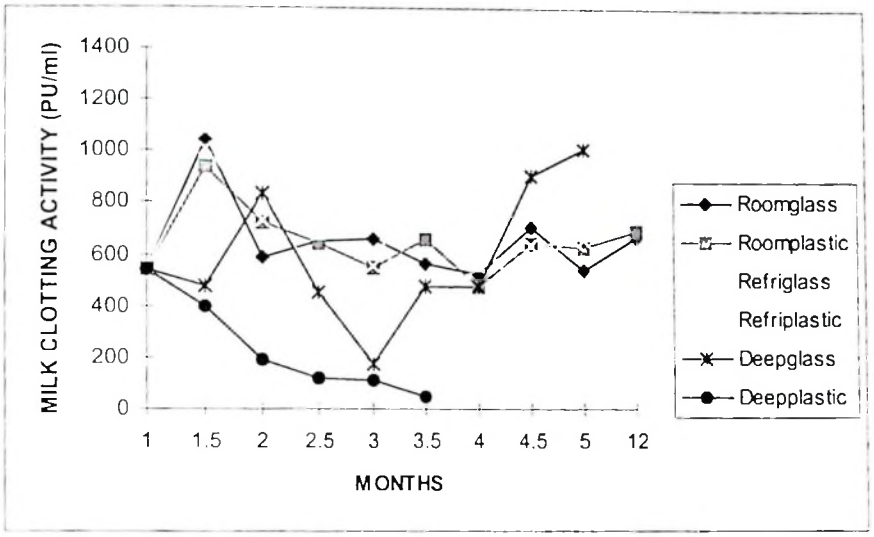
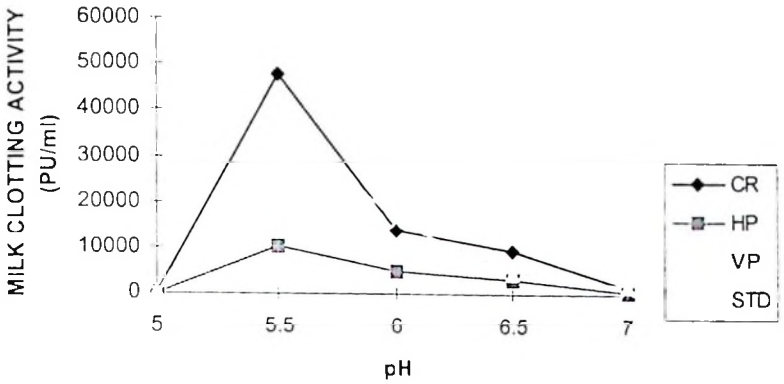
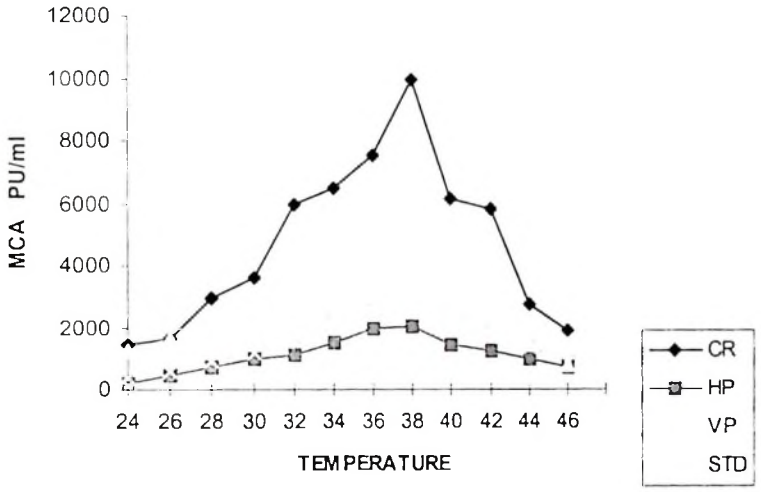


Figure 10: Effect of storage temperatures and type of container on MCA of VP

Effect of temperature and pH on MCA of extracts

Figure 11 shows that when MCA was determined at 30°C with milk of pH 6.76, all of the extracts were to some extent sensitive to pH 5.5-6.5 however, at pH 6.5-7.0 HP was very sensitive followed by STD then VP and finally calf rennet (CR). HP was more sensitive to temperature changes followed by STD, VP and then CR. All of them were stable at temperatures of 30-38°C.





Key: CR = (98%chymosin and 2% pepsin)

HP = Pepsin extracted by HCL

VP = pepsin extracted by vinegar

STD = 75% pepsin and 25% chymosin

Figure 11: The effect of change of milk pH and temperature on MCA of different coagulant preparations

Formagraph characteristics

Table 12 shows that the curd firmness rate K_{20} was faster with STD followed by VP, HP and finally LP. STD, HP and VP were not significantly different (P>0.05) for K_{20}. Also STD and VP for A_{30} were not significantly different (P>0.05) from each other but were significantly different from HP and LP. LP had the longest time of firming, lowest ratio of R/K_{20} and amplitude 30 min after gelling point.

Table 12: Formagraph milk coagulation characteristics with the clarified extract (min).

Extracts				Formograph characteristics (±se)	
	N	R	K_{20}	R/K_{20}	A_{30}
HP	4	15.5	18.0	0.86 (0.0) [a]	39.40 (0.8)[ab]
LP	4	15.0	20.0	0.75 (0.0) [b]	37.40 (0.8) [b]
VP	4	15.5	17.5	0.89 (0.0) [a]	40.60 (0.8) [a]
STD	4	15.0	17.0	0.88 (0.0) [a]	40.60 (0.8) [a]

Within column, means followed by the same letter do not differ significantly at (P>0.05) according to DMRT

Proteolytic activity of the extracts

The ratio of proteolytic activity to MCA was calculated to be $1:11.6 \times 10^2$, $1:12.1 \times 10^2$, $1:7.8 \times 10^2$ and $1:7.5 \times 10^2$ for HP, LP, VP extracts and STD respectively. The

protein breakdown by LP was higher followed by HP and finally VP which was close to that of the control.

TCA-soluble peptides released by HP and VP extracts from αs_1 casein

Table 11 shows that when the TCA filtrates after incubation of αs_1-casein, the NPN by optical density at 280 nm was 0.46, 1.50, 0.45 and 0.42 mg for HP, LP, VP and STD, extracts respectively. The Reverse Phase- FPLC chromatograms show peaks of peptides and proteins separated with acetonitrile gradient. The un-incubated sample A in Figure 12 contains presumably peptides and proteins present in the αs_1-casein. The number of peptides increased markedly in the incubated samples B and C and new peptides eluted after a short time. The arrows in A, B, C of Figure 12 mark the time of αs_1-casein fragments 1-23 which are the major peptides released by chymosin and pepsin. Differences in peptide profile of HP and VP show both specificity and activity between the extracts

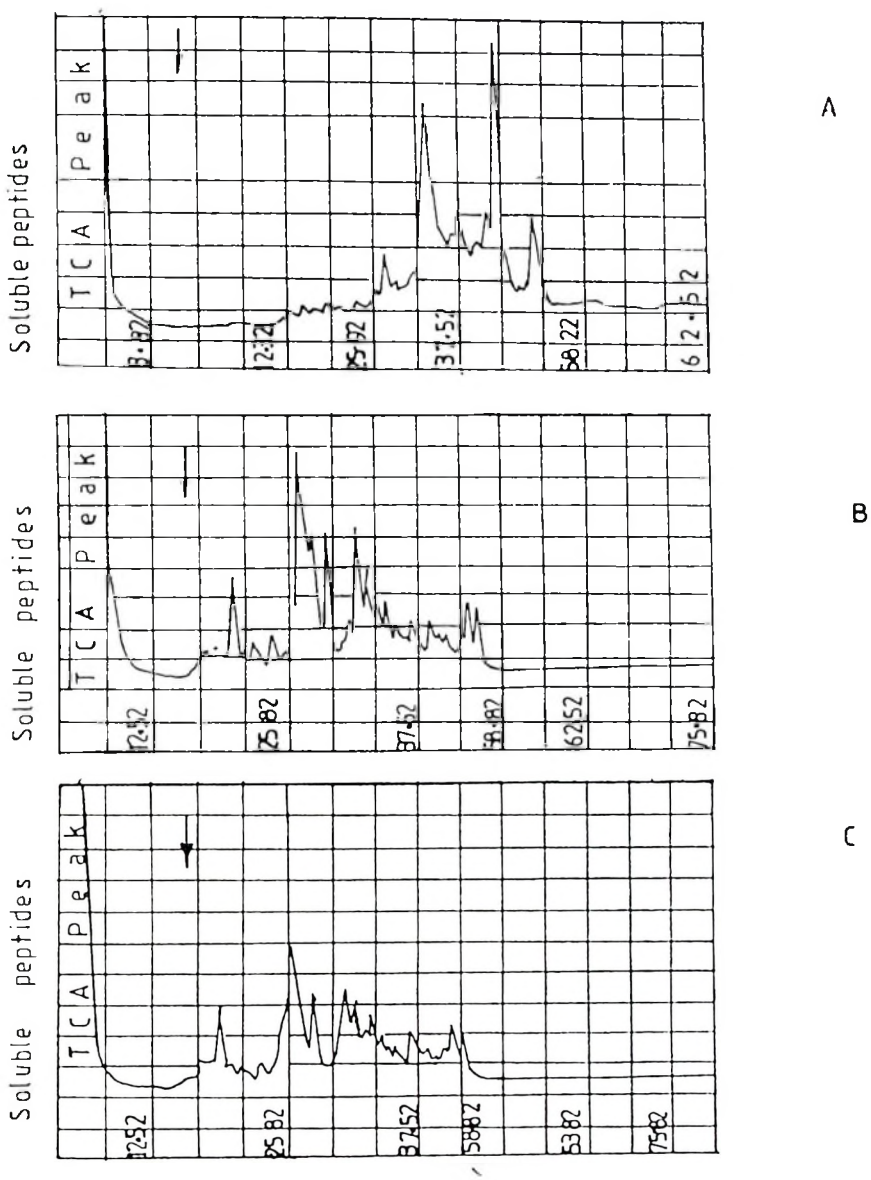
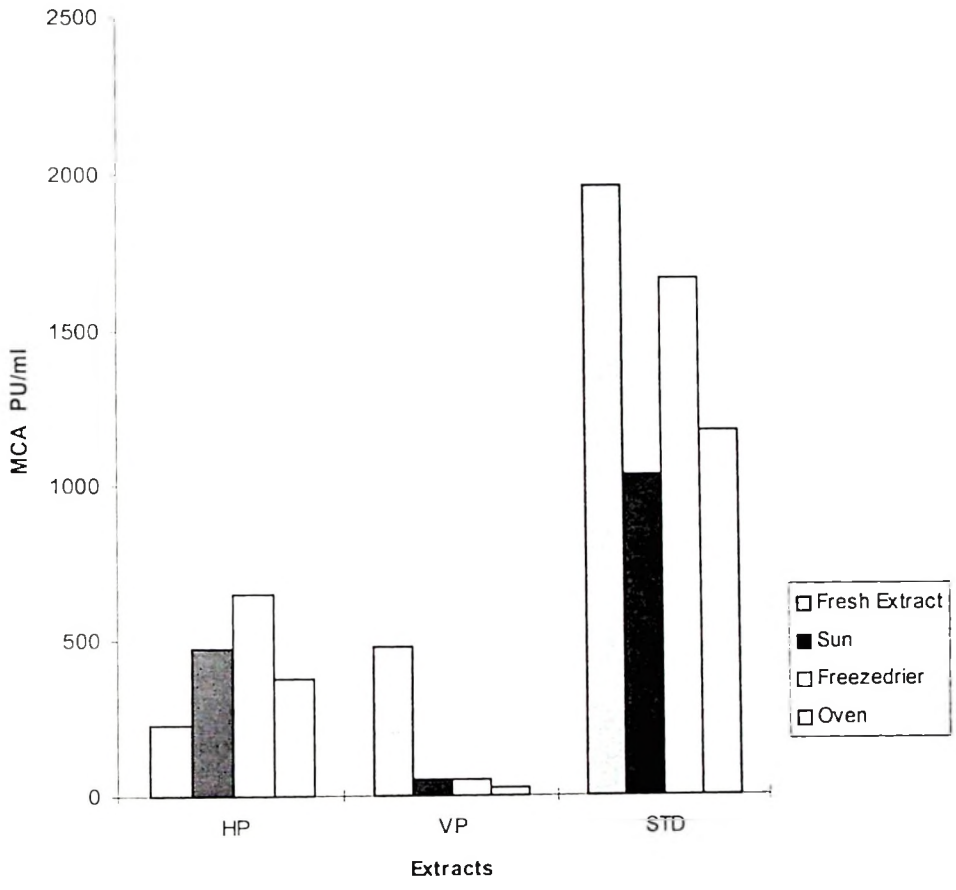


Figure 12: Separation by RP-FPLC of TCA soluble peptides released from αs_1-casein. A: un incubated control; B: HP extract incubated; C: VP extract incubated. The peptides separated were monitored at 214 nm, flow rate was 0.23 ml/min.

4.1.4 Effect of concentration methods

Figure 13 shows that when HP extract was concentrated by drying under sun light freeze drying or oven drying to constant weight, the enzymatic activity increase while VP extract lost 100 % of its activity. STD lost some activity with all the thre methods of concentration.



Note:

HP - pepsin by HCl

VP - pepsin by vinegar

STD – Control (75% pepsin and 25% chymosin)

MCA- Milk clotting activity

Figure 13: Clarified extract concentration methods and their milk clotting times (min)

4.2 Experiment 2: Purification of bovine pepsin

4.2.1 Purification on DEAE cellulose

Figure 13a shows that two peaks of milk clotting activity (MCA) were well resolved during the first step of purification of STD. Two MCA peak superimposed by AD peak were eluted as fractions number 28-30 and number 40-44. Since protein fractionation was only partial, further purification continued

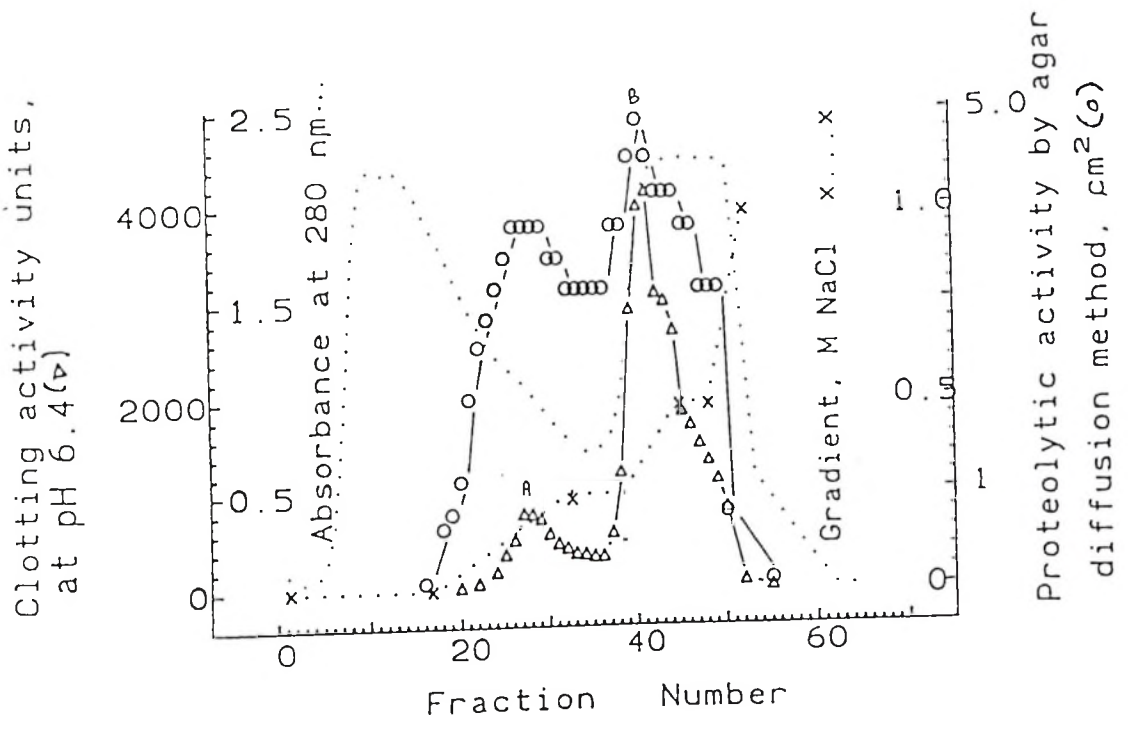


Figure 14a: Purification of standard rennet on anion exchanger Fast Q Sepharose column connected to FPLC, equilibrated with buffer A, loaded with 25 ml rennet which was eluted with buffer B (A + 2M NaCl), flow rate was 1 ml/min, fraction size 6 ml and 65 fractions were collected.

Step Ib: Purification of crude extract HP on DEAE- cellulose

Figure 14b shows that step 1 of purification of HP extract resolved two peaks of activities. MCA measured at milk pH 6.0 and 6.4 and proteolytic activity measured by agar diffusion method (AD) superimposed at fractions no. 21-29 and no.114-138. These peaks were expected to represent chymosin and pepsin, respectively. Formation of other peaks (OD_{280}) with no activity, indicated the presence of impurities. Therefore further purification to step II was continued.

The content of pepsin and chymosin

STD

As calculated from the MCA and volumes of pooled fractions, STD contained 25% chymosin and 75% pepsin

HP

HP extract contained 3.5% chymosin and 96.5% pepsin.

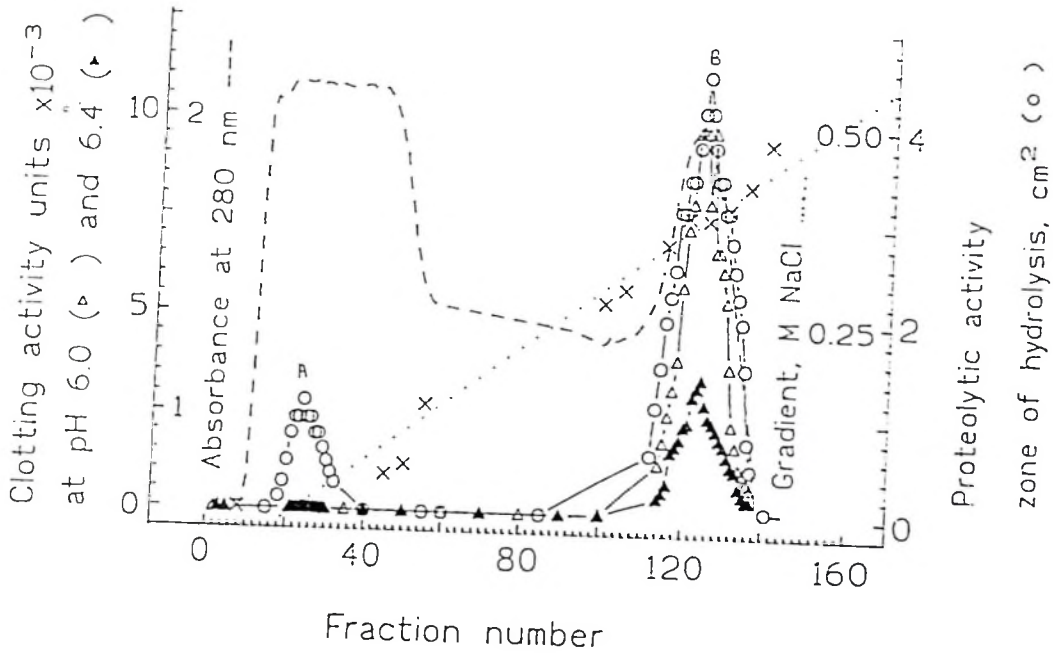


Figure 14b: Purification of HP by DEAE cellulose ion exchange chromatography. Column size 0.1 x 20 cm equilibrated with 0.025 M piperazine buffer pH 5.3, loaded with 25 ml extract, eluted by a linear gradient by mixing buffer A and B (A + 0.7M NaCl) at a flow rate of 1 ml/min, fraction size 4 ml and 140 fractions were collected

4.2.2 Step II: Purification of pepsin and chymosin by gel filtration

Pepsin and chymosin fractions from HP separated on DEAE cellulose were purified further by gel filtration chromatography. Pepsin fractions showed milk coagulating active peak at fractions 68-75 (Figure 15a). Chymosin fractions showed milk coagulating active peak at fractions 85 - 90 (Fig.15b)

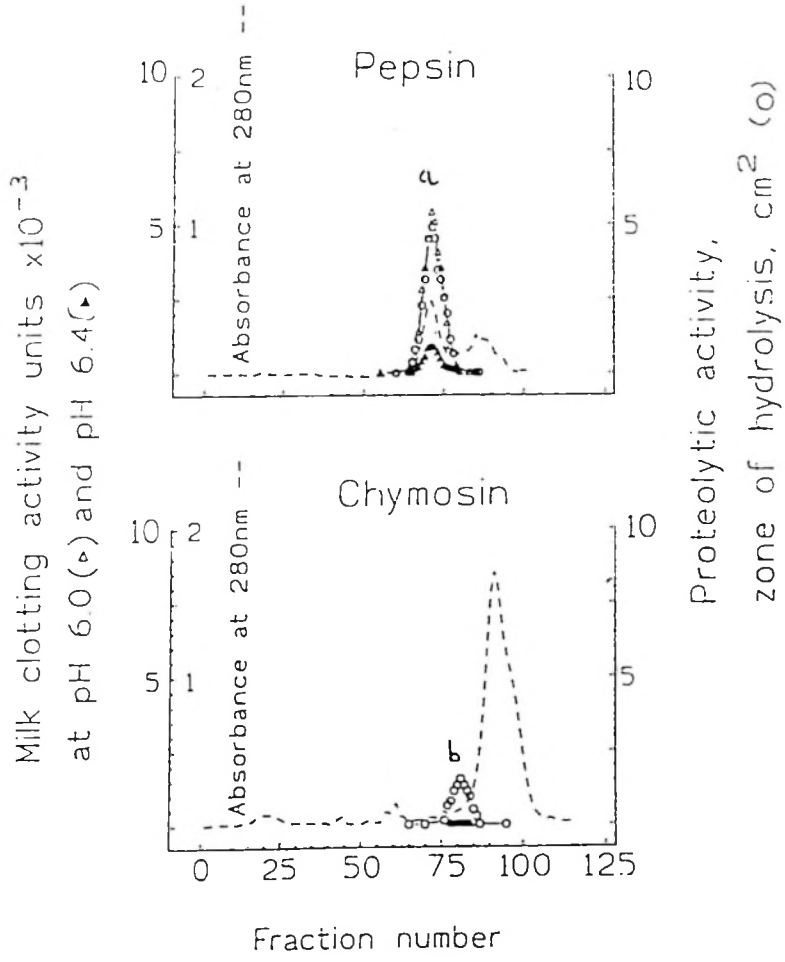


Figure 15: Purification of pepsin and chymosin fractions by gel filtration chromatography on Ultrogel. Column (size 2.1 x 120 cm) was equilibrated with buffer A containing 0.15 M NaCl, the same buffer was used for separation of samples, flow rate 1 ml/ min, fraction size 4 ml and 98 fractions were collected

4.2.3 Step III: Purification of pepsin by Mono Q ion exchange chromatography

The pepsin fractions pooled from the Ultrogel column were equilibrated with buffer A and separated on a Mono Q column. The highest MCA and AD was found in fractions 18-19 indicating that pepsin was at this stage well purified. Chymosin was purified in the same way as pepsin and a small single peak emerged (Fig.16)

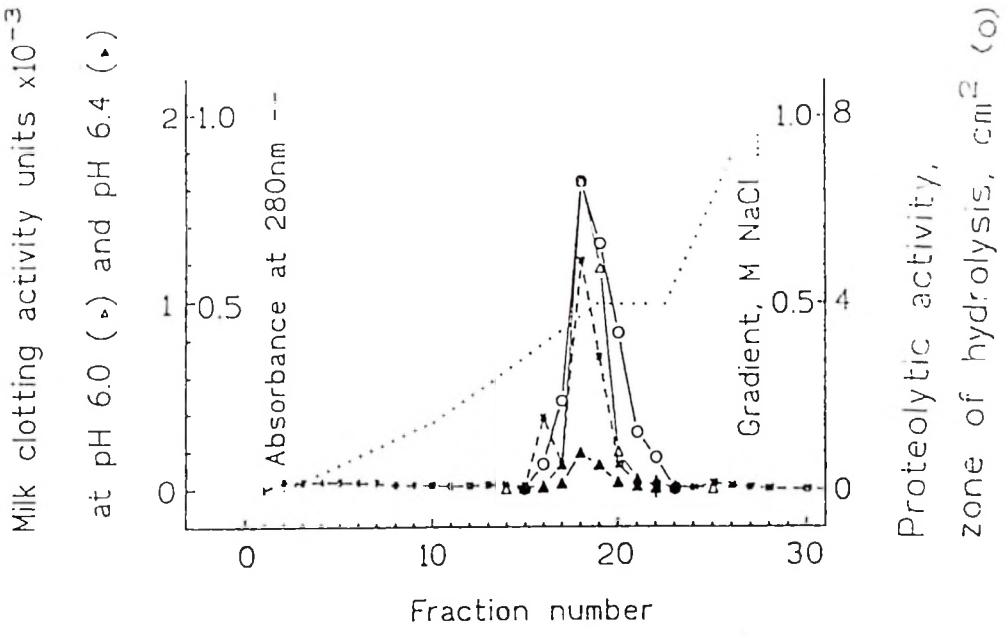


Figure 16: Purification of pepsin by Mono Q ion exchange chromatography using FPLC equipment. Column size 0.5 x 5 cm was equilibrated with buffer A and loaded with 12 ml solution pooled from step II, eluted with a linear gradient of buffer B(A + 2M NaCl), flow rate 0.5 ml/ min, fraction size 1.5 ml and 36 fractions were collected.

4.2.4 Step IV: Purification of pepsin fractions on Phenyl Sepharose.

In step IV the highest OD_{280}, MCA and AD superimposed at fractions 22-26. Figure17 shows that pepsin collected from Mono Q was eluted from Phenyl Sepharose as three partially separated peaks of protein marked A, B and C. All the 3 peaks had MCA and AD activities. Pepsin fractions were eluted from the column at the end of the gradient when the ammonium sulphate concentration was reduced to 0.2M. A moderate peak without MCA and AD was eluted before the main protein peak.

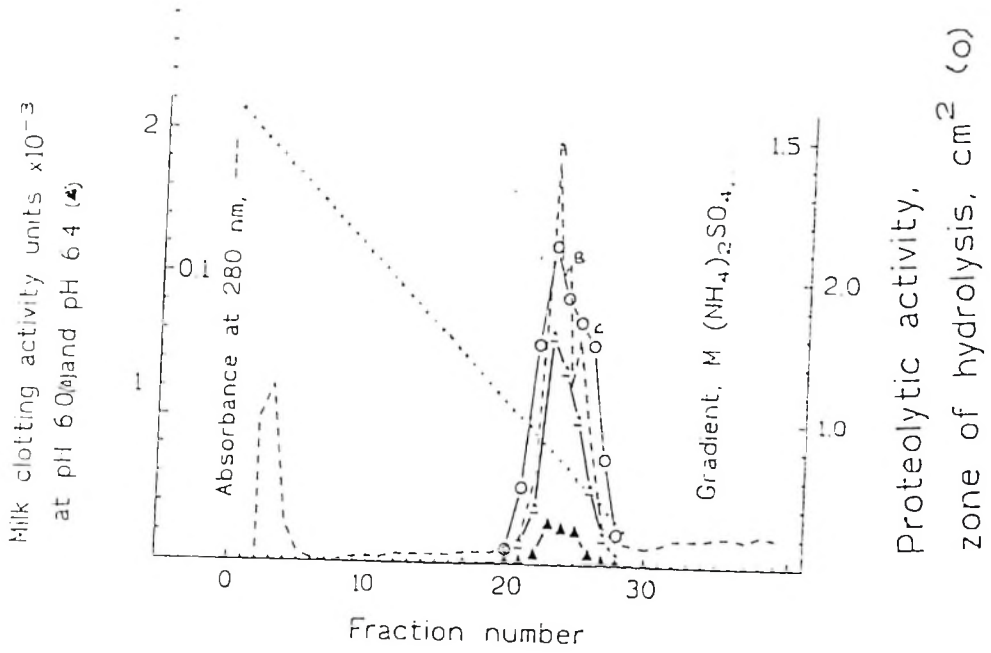


Figure 17: Purification of pepsin fraction HP on Phenyl Sepharose using FPLC equipment. Column type HR5/10 was equilibrated with piperazine buffer A containing 1.7 M ammonium sulphate loaded with 0.5 ml extract pooled from step III, the column was eluted with a linear gradient of buffer A to B(B was buffer A without ammonium sulphate) with flow rate 0.4 ml/min and 40 fractions were collected.

4.2.5 Step V: The rechromatographed pepsin peak A from Phenyl sepharose

The rechromatographed pepsin peak A from step IV on Mono Q showed a single symmetrical peak of protein indicating that pepsin fraction was homogeneous.

4.2.6 Pepsin recovery during purification steps

Table 13 shows that as the crude extract is purified stepwise, the amount of protein in the sample to be injected into the column, TP, total MCA and recovery decreased. MCA units, specific activity and fold purification increased. Pepsin was purified 366 fold, a large proportion (32.7%) of purified enzyme was recovered. Specific activity expressed as MCA /mg protein (OD_{280}) increased steadly through the purification steps.

Table 13:Summary of pepsin purification steps

Steps	Volume	MCA	OD_{280}	TP mg/ml	Total MCA	Specific activity	Fold puri	Reco-very %	Reco-Very (mg)
Crude [1]	25	24	2.3	56.3	600	11	1	100	56.3
DEAE	4	50	1.9	7.7	200	26	2.4	33.3	13.7
Gel	4	92	0.5	2.0	368	185	16.8	61.3	3.6
Mono Q	1.5	175	0.5	0.7	262	350	31.8	43.7	1.2
Phenyl	0.5	392	0.1	0.1	196	3920	356	32.7	0.2

Key:

[1] Crude HP extract DEAE cellulose Gel Filtration Mono Q Phenyl Superose

TP = Volume x OD (mg protein/ml) Total MCA = Volume x MCA

Specific activity = MCA/OD expressed as MCA/mg protein

Recovery % = Total MCA of each step of purification divided by original MCA multiplied by 100

Recovery in mg = TP in each step divided by initial TP

Fold purification = ratio of specific activity of that step to the activity of the original extract

4. 2.7 Quality of purified enzyme

Homogeneity by chromatographic behaviour

Figure 18: shows the chromatographically symmetric peak indicating that mainly one protein made up the peak.

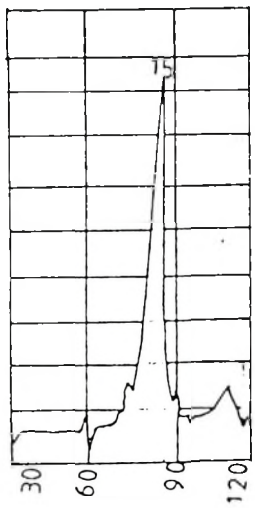


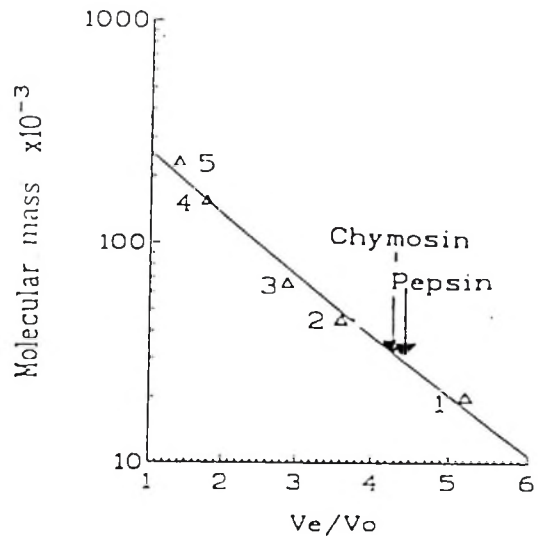
Figure 18: Rechromatographed pepsin peak A on Phenyl Superose from step IV. Fifty microlitre fractions pooled from sub peak A of Figure 16 was rechromatographed on Mono Q. The Figure shows a reprint of intergrator record.

Absence of other enzymes

Figure 18 had maximum MCA at one peak, unlike in Figure 16 where small peaks were seen but they had no activity indicating that the sample still contained some impurities.

Molecular mass of pepsin and chymosin eluted by gel filtration

Figure 19 shows that both pepsin and chymosin are eluted between molecular mass protein markers 20 and 45 κDa.



Molecular mass (MM) of protein markers

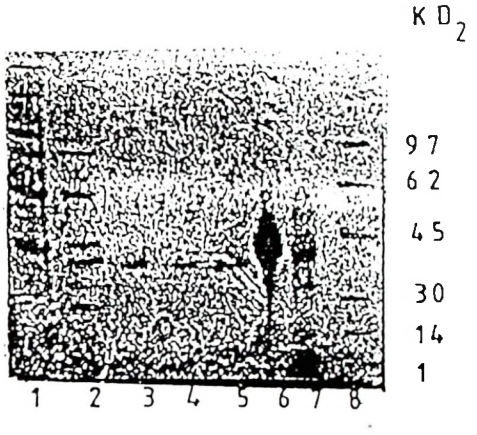
1. Trypsin inhibitor	MM 20,000
2. Egg serum albumin	MM 45,000
3. Bovine serum albumin	MM 66,000
4. Aldolase	MM 150,000
5. Catalase	MM 232,000

Figure 19: Standard curve for determination of MM where STD proteins were eluted in gel filtration chromatography (Ultrogel). Vo = Void volume and Ve = elution volume for the STD (above)

The molecular mass for pepsin and chymosin calculated from the standard curve were 34 and 34.5 κDa (κDa=1000 Da) respectively.

SDS-PAGE for fractions pooled from purification steps

Figure 20 shows the diagram for determination of molecular mass for pepsin purified by DEAE cellulose, Gel filtration and Mono Q chromatography. Single well separated bands of purified pepsin (marked 4 and 5) from Mono Q and Phenyl Sepharose samples were observed to be between 30 and 45 kDa.



Key

1. Molecular mass protein marker
2. Dialysed and concentrated sample
3. Pepsin from DEAE cellulose
4. Pepsin from mono Q
5. Pepsin from phenyl sepharose
6. chymosin from DEAE cellulose (HP)
7. Chymosin from DEAE cellulose (STD)
8. Molecular mass protein markers

In position 2 pepsin was faintly observed just below chymosin

In position 7 was chymosin from DEAE cellulose (STD)

Figure 20: Electrophoregram of samples from all steps of purification. Sample used 50 µl and sample applied was 3µl

Amino acid N-terminal sequence of pepsin samples

The Swiss-Pron. Data Base found that the N-terminal sequences corresponded to 7 segments of pepsinogen and it is apparently the N-terminal sequence of pepsin A. The identified sequences were as follows:

Mono Q sample.... Ile-gly-phe-leu-gly-asp-tyr-leu

Phenyl superose.sample...Ile-gly-phe-leu-gly-asp-tyr-leu

Both Mono Q and rechromatographed Mono Q sample on Pheny Superose had the same N-terminal amino acid sequence, indicating good purity after purification on Mono Q.

4.3 Experiment 3: Cheese quality assessment of HP and VP extracts

4.3.1 Renneting times of cheese milk, total nitrogen and butterfat in whey for SUA Alpine and SUA Pasta

Renneting times (RT)

Data for RT of all the enzyme combinations are summarised in Table 14

The RT for SUA Alpine cheese milk ranged from 40 (100CR) to 60 (75VP). RT increased with increasing relative amount of pepsin for all these cheeses. VP combinations had slightly longer RT than HP combinations. RT for SUA Pasta cheese milk ranged from 44 - 50 min and the control used 45 min. 100HP, 75HP, 25HP and 50VP cheeses had similar RT to the controls. For SUA Pasta, RT was not increasing with increasing level of pepsin but the range was small between combinations.

Total nitrogen (TN)

TN in SUA Alpine whey ranged from 0. 49% for 25HP to 0.66% for 100VP and the control had 0.52%, (Table 14). TN loss in whey was increasing with increase of pepsin level. VP combinations were loosing slightly more TN in whey than HP combinations where 100VP treated cheese milk had the highest. TN in SUA Pasta whey ranged from 0.48% for 100CR to 0.92 for 100VP, the control cheese had the lowest TN. Most of the VP combinations had slightly higher TN in whey, but 25VP and 100VP had the highest TN loss and were significantly different ($P<0.05$) from others.

BF in whey

BF loss in SUA Alpine whey ranged from 0.20% for 50HP and 75VP to 0.60% for 100VP. The control had 0.50% BF. Also BF loss was slightly more in VP combinations than HP. Loss of BF in SUA Pasta ranged from 1.7% for 50VP to 3.2% for 100VP (BF in SUA Pasta include BF from the whey before and after moulding cheese in hot water). The control lost 2.5.0%. HP combinations lost slightly more BF in whey than VP but 100VP was the highest in loosing BF and TN in whey (Table 14).

Note: RT, TN and BF were observed for cheese made at SUA laboratory where the procedure and manufacturing conditions were well controlled and the sample storage facilities were available as compared to village conditions without.

Table 14: Renneting times (RT) of cheese milk, total nitrogen (TN) and butterfat (BF) in whey for the production of SUA Alpine and SUA Pasta.

Cheese	Variable	Rennet/ pepsin combinations								
		100R	100HP	25HP	50HP	75HP	100VP	25VP	50VP	75VP
SUA alpine	RT	40 ±1^{d}	58 ±1^{ab}	41 ±1^{d}	43 ±1^{cd}	45 ±1^{c}	55 ±1^{b}	43 ±1^{cd}	45 ±1^{c}	60 ±1^{a}
SUA pasta	RT	45 ±1^{b}	45 ±1^{b}	46 ±1^{b}	50 ±1^{a}	45 ±1^{b}	45 ±1^{b}	44 ±1^{b}	45 ±1^{b}	50 ±1^{a}
SUA alpine	TN	.52 ±. 001^{cd}	.55 ±.001^{c}	.49 ± .001^{d}	.50 ± .001^{d}	.52 ± .001^{cd}	.66 ±.001^{a}	.55 ± .001^{c}	.62 ± .001^{b}	.64 ± .001^{ab}
SUA pasta	TN	.48±.01^{e}	.56 ±.01^{cd}	.59 ±.01^{c}	.59 ±.01^{c}	.48 ±.01^{e}	.92 ±.01^{a}	.69 ±.01^{b}	.54 ±.01^{d}	.66 ±.01^{b}
SUA alpine	BF	.50 ±0.01^{b}	.40 ±0.01^{c}	.30 ±0.01^{d}	.20 ±0.01^{e}	.50 ±0.01^{b}	.60 ±0.01^{a}	.50 ±0.01^{b}	.40 ±0.01^{c}	.20 ±0.01^{e}
SUA pasta	BF	2.5 ±.11^{bc}	3.0 ±.11^{ab}	2.7 ±.11^{b}	2.2 ±.11^{cd}	2.0 ±.11^{c}	3.2 ±.11^{a}	2.0 ±.11^{c}	1.7 ±.11^{f}	2.0 ±.11^{c}

Within the row, means followed by the same superscript do not differ significantly at P>0.05 according to DMRT

Key of Tables

BF = butterfat
MC= moisture
TN = total nitrogen
WSN= water soluble nitrogen
RI= ripening index **DRMT = Duncan's Multiple Range Test**
Superscripts on SPC, coli and YM represents the counts in ifu/ml in cheese

4.3.2 Chemical characteristics of the cheeses

pH of the cheese

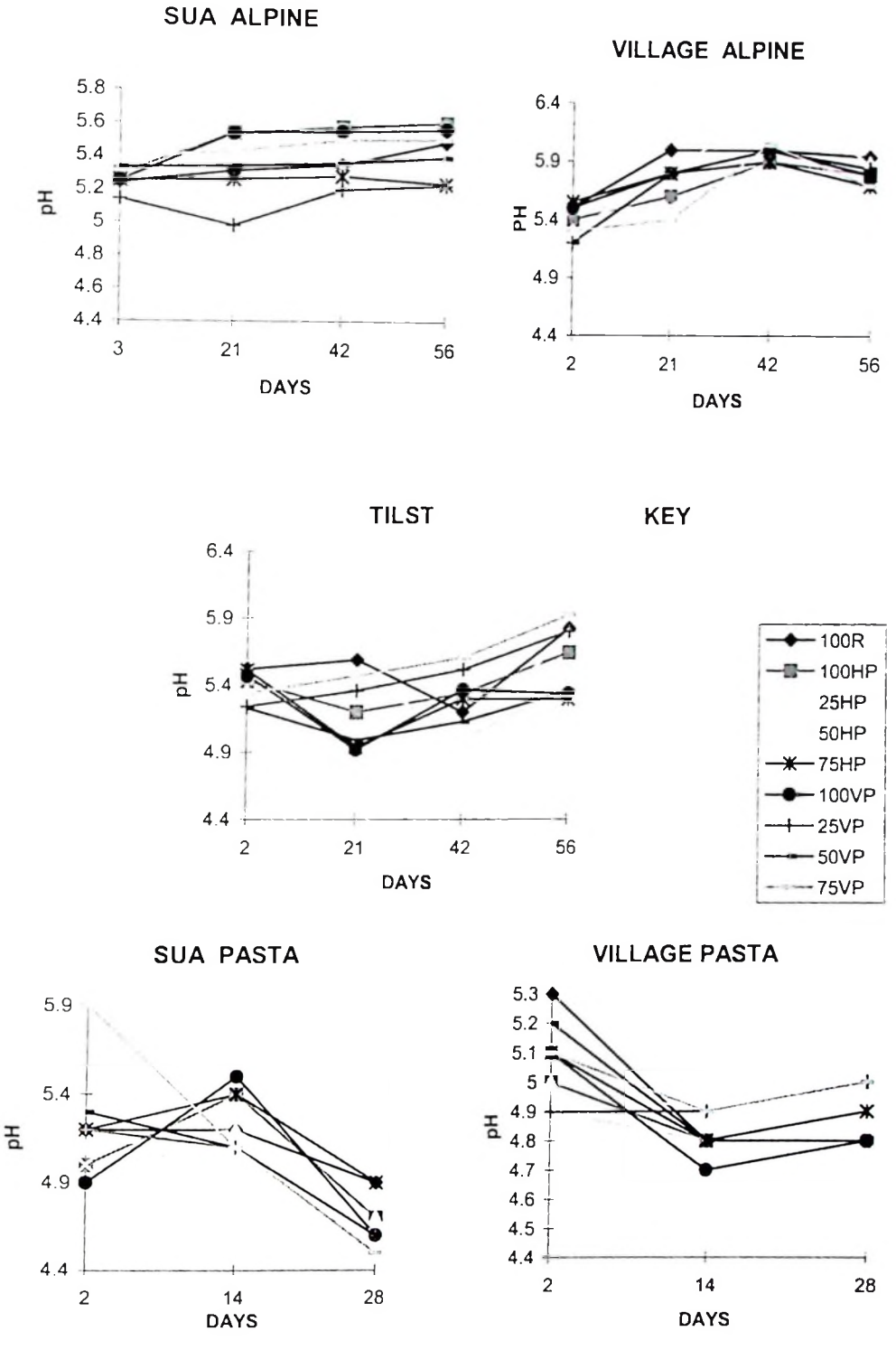
The trend of pH for all types of cheese made with different enzyme combination are shown in Figure 21 and data at maturity are summarised in Tables 15- 19.

The pH of most SUA Alpine cheeses was increasing with days of ripening except that cheese 25HP, 50HP and 25VP had lower pH at 21 days and then continued increasing to 56 days. The range of pH at 42 days which is the right maturity time, was 5.2 to 5.6 for cheese 25VP and 100HP, respectively, where the control 100CR had pH 5.4.

In village Alpine cheeses, the pH increased with ripening time to 42 days and then started decreasing to 56 days. At 42 days the range was 5.85 to 6.36 for cheese 75HP and 25 HP, respectively, and the control had pH 5.9.

The pH among Tilsiter cheeses varied a lot some were increasing with ripening time while others were decreasing and then increasing to 56 days. The pH range at 42 days was 5.0 to 5.6 for cheese 50HP and 75VP, respectively, and the control had pH 5.2. In some SUA Pasta cheese, pH increased while in others it decreased up to 14 days and then decreased in all of them to 28 days. The range of pH at 14 days which is the correct ripening time was 5.1 to 5.5 for cheese 75VP and 100VP, respectively, and the control cheese had pH 5.2

In all village Pasta cheese, the pH decreased up to 14 days and some continued with the same pH while in some cheeses it increased slightly up to 28 days. Range of pH at 14 days was 4.7 to 4.9 for cheese 100VP and 25HP, respectively, and the control had pH 4.8.



Key

X axis = pH values of cheeses
Y axis = Days of cheese ripening

Figure 21: The pH values of cheeses made with different combinations of CR/pepsin during cheese ripening times of 28 or 56 days.

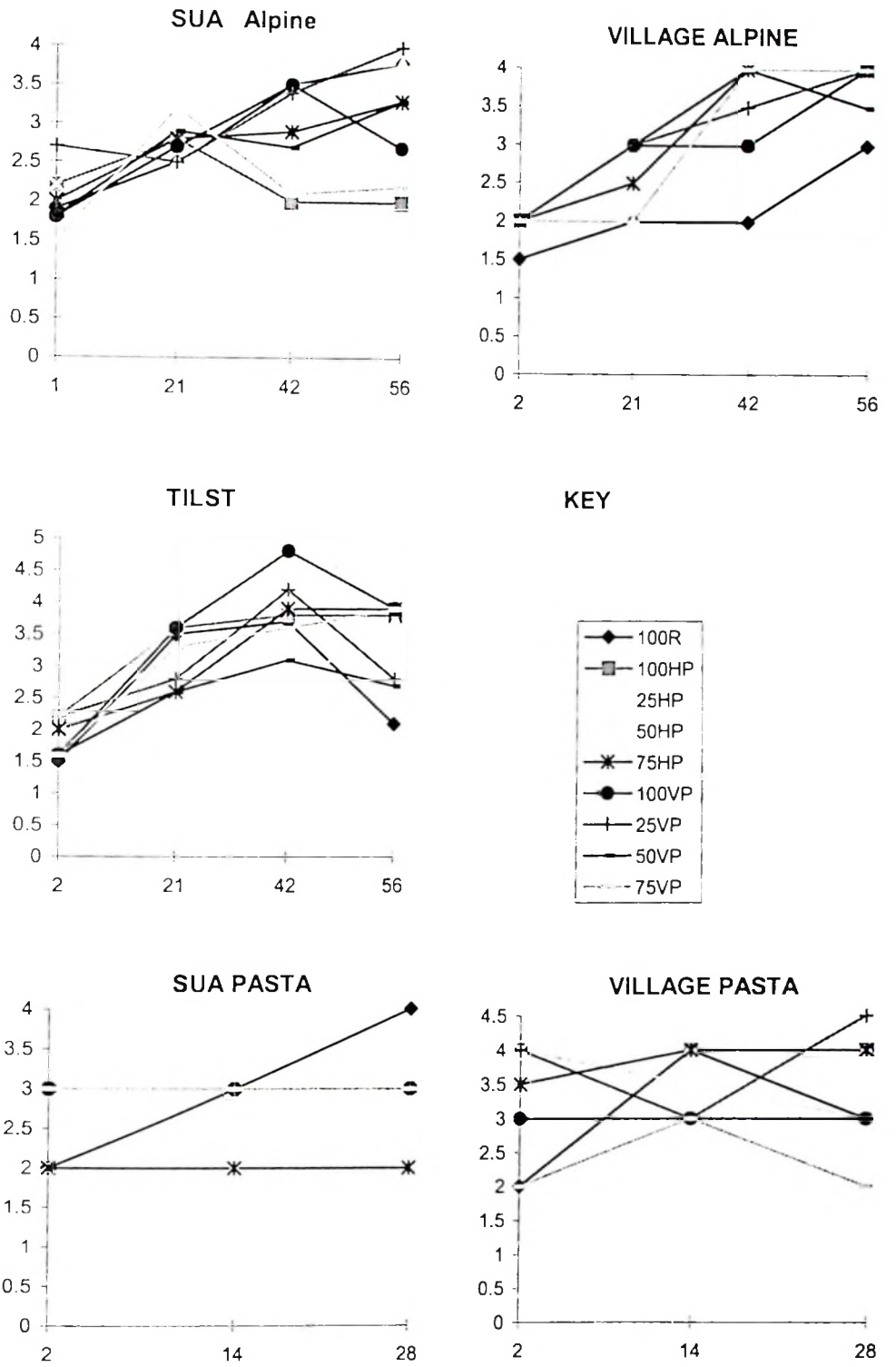
Cheese Acidity

The trend of acidity for all types of cheese made with different enzyme combination are shown in Figure 22 and data at maturity are summarised in Tables 15-19. In most SUA Alpine cheeses acidity increased up to 56 days except cheese 75VP and 100HP which decreased at 42 days and maintained at the same level up to 56 days. Acidity at 42 days ranged from 2.0 % to 3.6% for cheese 100HP and 25HP, respectively, and the control had 3.5%. Acidity in all village Alpine cheese increased up to 56 days of ripening. At 42 days the pH ranged from 2.0 % to 3.8 % for cheese 100CR and 75HP respectively.

In all the Tilsiter cheeses acidity increased up to 42 days and then decreased up to 56 days in all the cheeses except for cheese 25HP in which the acidity decreased up to 42 days and then increased up to 56 days. At 42 days the range of acidity was 2.7% to 5.0 % for cheese 25HP and 50HP, respectively, and the 100CR had 3.7%.

Acidity in most SUA Pasta cheeses did not change with ripening time except the acidity in cheese 100CR and 25HP which increased from 2 days and 14 to 28 days of ripening time respectively. Range of acidity at 14 days was 1.6 % to 3.1% which were 75HP and 75VP cheeses and 3.1% for the control cheese.

In village Pasta cheeses, the acidity varied a lot among cheeses in which some decreased, some decreased and increased again while others maintained the same level of acidity up to 28 days. The range of acidity at 14 days was 2.7% for cheese 75VP to 4.4% for cheese 100HP. The control had 4%.



Key : X axis = Percent acidity in cheese
Y axis = Days of cheese ripening

Figure 22 : Acidity (%) changes in cheeses made with different combinations of CR/pepsin during 28 or 56 days of ripening

Moisture content of cheese

The changes in moisture content for all types of cheese made with different enzyme combination is shown in Figure 23 and data are summarised in Tables 15-19.

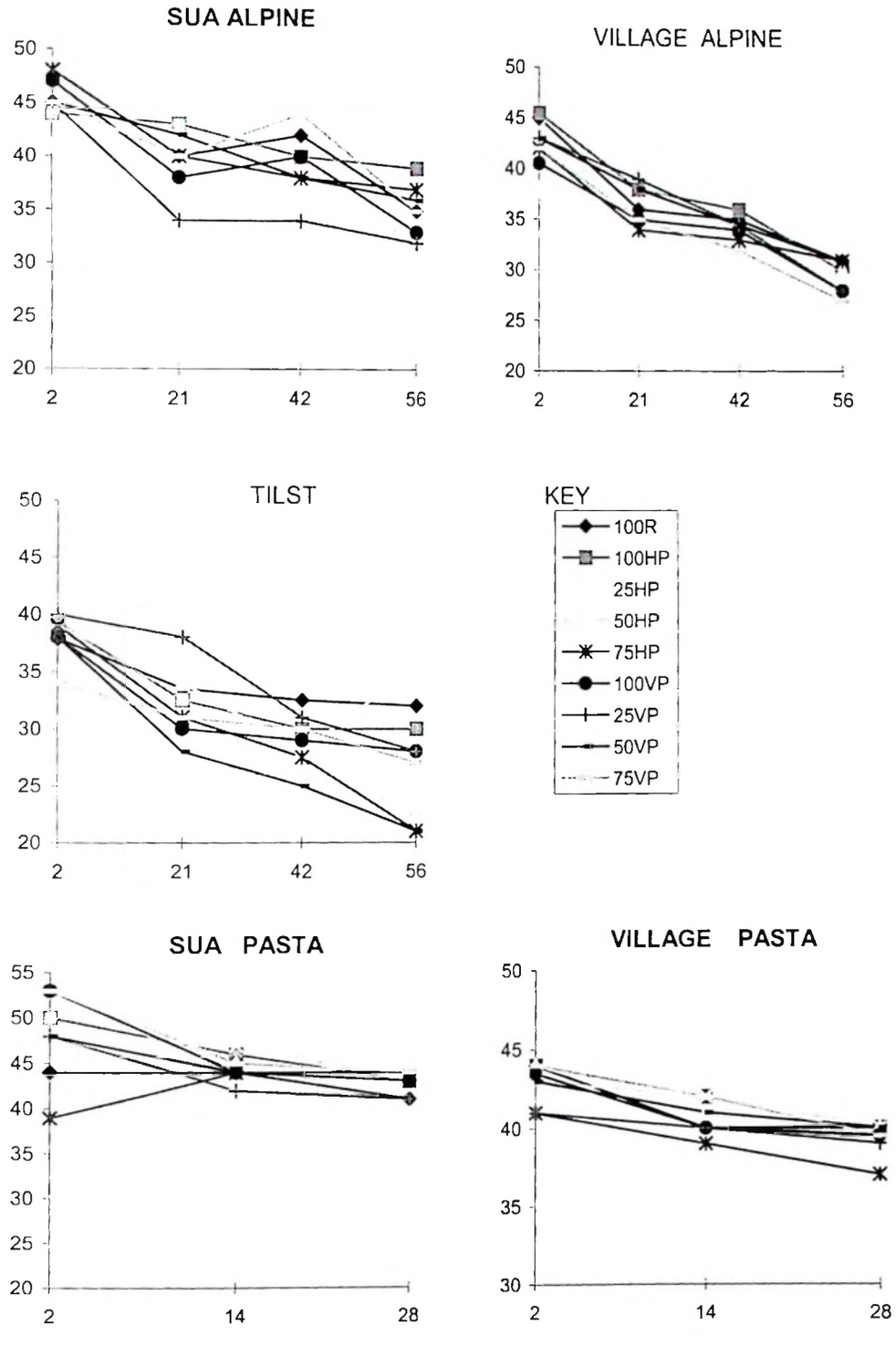
In all the SUA Alpine cheeses, moisture decreased up to 56 days but for cheese 75VP, 100CR and 100HP moisture increased slightly between 21 and 42 days and then decreased up to 56 days. Moisture at 42 days ranged from 32% for cheese 25HP to 44 % for 75VP, and control cheese had 41.6 %. Cheese 25HP, 25VP and 50HP were loosing moisture more than others.

Moisture in all the village Alpine cheeses decreased up to 56 days. The range at 42 days was 32, 36% for cheese 75VP and 100HP, respectively, and 100CR had reached 35%, and cheese 25HP had lost more moisture than the others.

In all the Tilsiter cheeses, moisture decreased with ripening time except that for cheese 25HP moisture increased from 42 to 56 days. Moisture at 42 days ranged from 26 - 38% for cheese 50VP and 25VP, respectively, where the control had 33%.

Moisture in all the SUA Pasta cheese decreased up to 28 days. The range at 14 days was 42% for cheese 25VP to 49% for 50HP and the control had 44%.

For all the village Pasta cheeses, moisture decreased up to 28 days, and at 14 days it ranged from 37 - 44% for 75HP and 100HP, respectively.



Key X axis = Moisture content in cheese
Y axis = Days of cheese ripening

Figure 23: Moisture changes in cheeses made with different combinations of rennet/ pepsin during ripening time of 28 or 56 days.

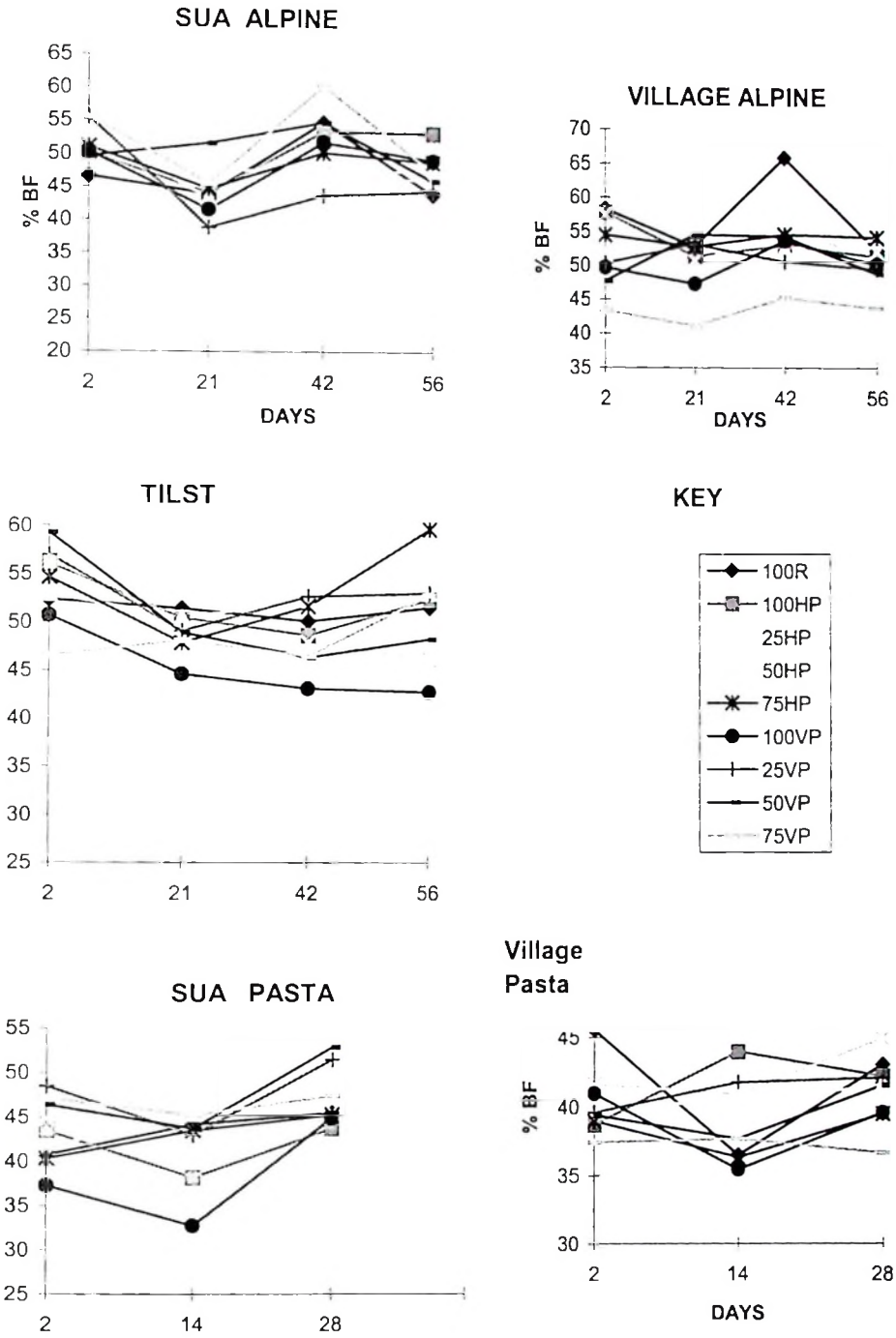
Butterfat (BF) in DM

The trend of percent BF for most cheeses was decreasing with ripening time. The BF at 42 and 14 days which were the right time of cheese consumption depending on the type of cheese are shown in Figure 24 and data at maturity are summarised in Table 15-19. At 42 days of maturity of SUA Alpine cheeses, the butterfat content ranged from 43.7 % to 60.3 % for cheese 25VP and 75VP, respectively, and the control had 54.8%.

For the Village Alpine cheeses at maturity the BF ranged from 45.3 - 67.0% for 75VP and 100CR, respectively. For the Tilsiter cheeses at maturity the BF ranged from 43.1 - 52.7% for cheese 100VP and 25VP respectively, and the control cheese contained 50.1%

At 14 days, the BF of SUA Pasta ranged from 32.8 - 48.1 % for 100VP and 50HP, respectively, and the control cheese had 44.2%. Cheese 100VP and 100HP had much lower values of BF than the others.

At maturity (14 days) of village Pasta cheeses, the BF ranged from 35.4 - 44.5% for cheese 100VP and 100HP, respectively and the control had 36.5%.



Key : X axis = Percent butterfat in cheese (BF)
Y axis = Days of cheese ripening

Figure 24: The BF values of cheese made with different combinations CR/pepsin during 28 or 56 days of ripening

NaCl content of cheese (DM)

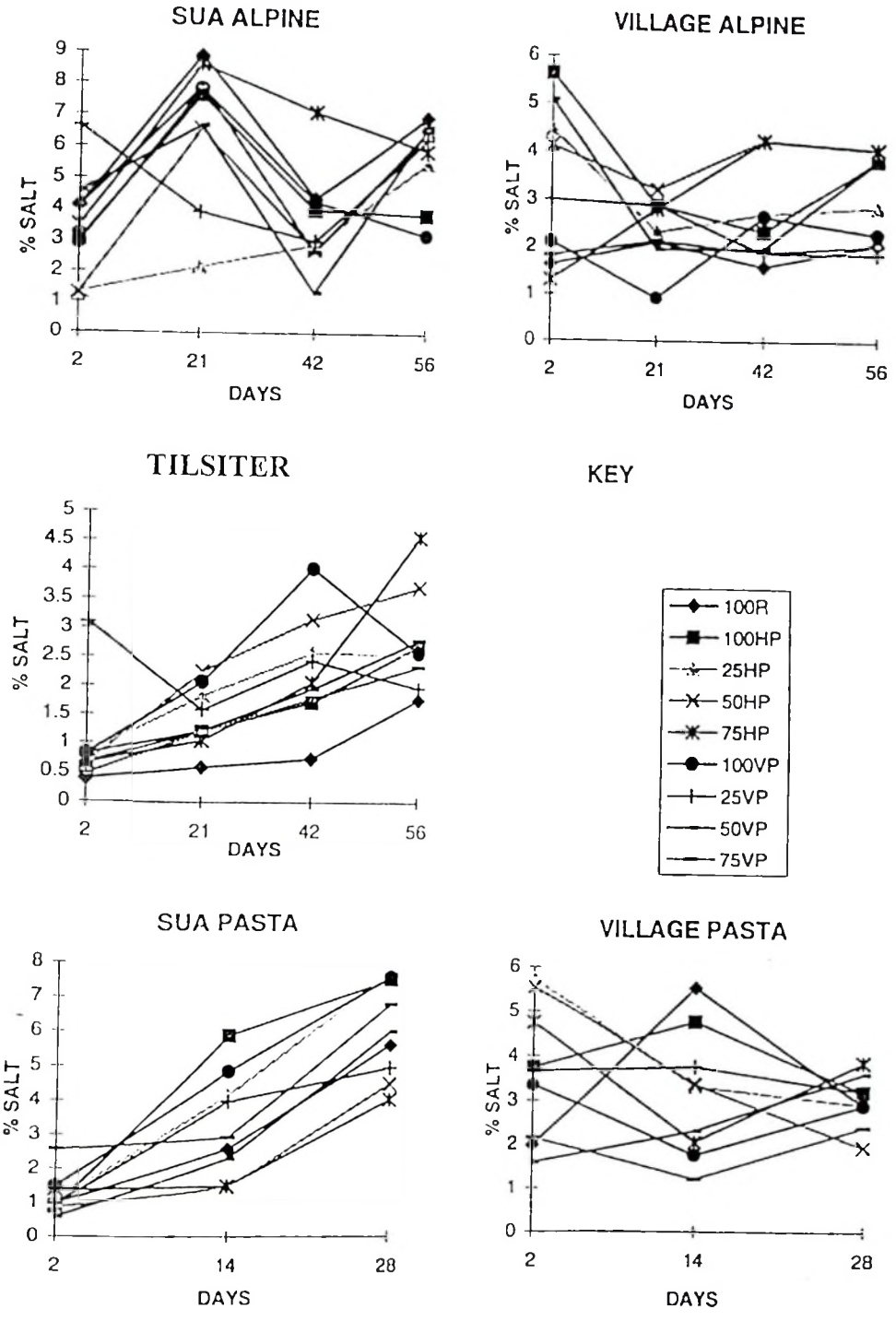
The trend of NaCl content for all the types of cheese made with different enzyme combination are shown in Figure 25, and data at maturity are summarised in Table 15-19. NaCl in most SUA Alpine cheeses increased up to 21 days, then decreased up to 42 and thereafter it increased in some cheeses and decreased in others. In cheese 75HP after it had increased to 21 days it decreased slowly to 56 days, while in cheese 50VP it decreased from day 2 to 42 days and then increased up to 56 days, and in cheese 25HP the increase lasted all the time to 56 days. Thus NaCl varied a lot among cheeses with a range at 42 days of 1.3 - 7.1% for cheese 50VP and 75HP, respectively, and the control had 4.5 %

NaCl varied a lot among village Alpine cheeses during ripening. At 42 days NaCl in cheese ranged from 1.6 - 4.3 % for cheese 100CR and 50HP, respectively.

In most Tilsiter cheeses, NaCl increased with ripening time up to 56 days. At 42 days NaCl ranged from 0.7 % to 4.1% for cheese 100CR and 100VP, respectively. Chesees 25VP experienced a small decrease in NaCl content over the whole period.

In all the SUA Pasta cheeses, NaCl increased much with ripening time. At 14 days the range between cheeses was 1.5 - 5.9% for cheese 50HP and 100HP, respectively, and the control cheese had 2.6 %.

NaCl in village Pasta cheese varied a lot, in some cheeses NaCl increased up to 14 days then decreased up to 28 days while in others it was the opposite. Range at 14 days was 1.2 - 5.5% for cheese 75VP and 100CR, respectively.



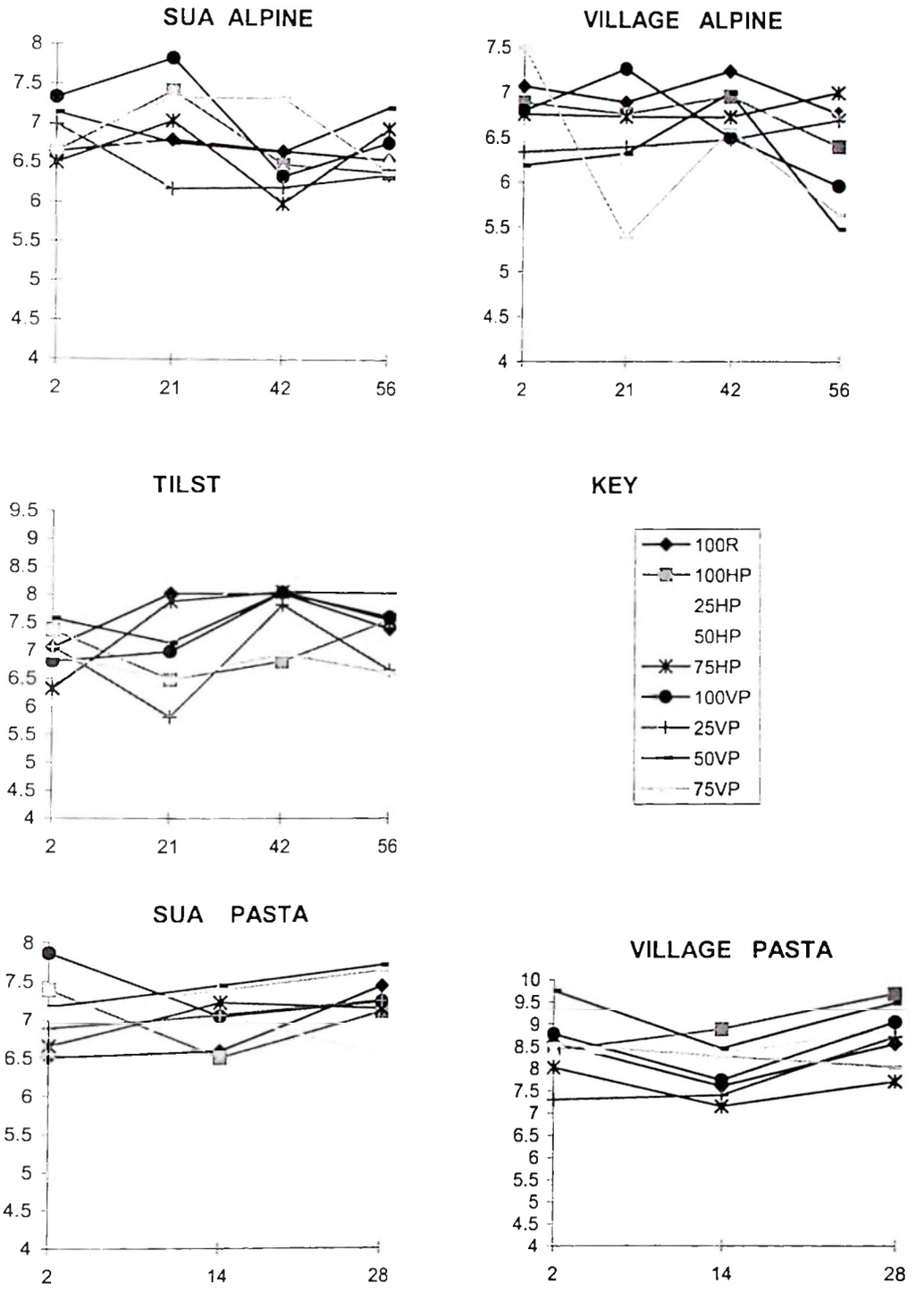
Key
X axis = Percent NaCl
Y axis = Days of cheese ripening

Figure 25: NaCl content in cheeses made with different combinations of CR/pepsin during 28 or 56 days of ripening

TN in cheese

The trend of TN for all types of cheese made with different enzyme combination are shown in Figure 26 and data at maturity are summarised in Table 15-19. TN in SUA Alpine cheeses increased up to 21 days then decreased up to 42 days and in some TN increased again up to 56 days while in others it decreased up to 56 days. At 42 days TN ranged from 6.0 - 7.4 % for cheese 75HP and 75VP, respectively, and the control had 6.9% (DM). In all the village Alpine cheeses TN was of the same trend. It slightly decreased up to 21 days, then slightly increased up to day 42, and then decreased again up to day 56 except for cheese 75VP and 100VP which had opposite trends with decreasing TN up to 21 days and then increasing up to 56 days while for 100VP TN increased up to day 21 and then decreased up to day 56. Range at 42 days was 6.3% for 25HP to 7.3% for the control. In most Tilsiter cheeses, TN increased up to 42 days while in a few cheeses it decreased up to 21 days and then increased up to day 42 and in all, cheese 100HP TN decreased up to day 56. Range at 42 days was 6.8% for 100HP to 9.1% for 25HP, while the control had 8.0%

In most SUA Pasta cheeses, TN increased with ripening time up to 28 days except cheese 100VP and 100HP in which TN decreased up to 14 days and then increased up to day 28 and for 50HP and 75HP in which TN decreased from 14 to 28 days. Range at 14 days was 6.5% for 100HP to 7.5% for 50VP, and the control had 6.6%. TN in most village Pasta cheese decreased up to 14 days and then increased up to day 28 except in cheese 75VP where TN decreased from 14 to 28 days. Range at 14 days was 7.1% for 75HP to 8.9% for 100HP, and the control had 7.6%.



Key
X axis = Percent total nitrogen
Y axis = Days of cheese ripening

Figure 26: Total nitrogen in cheeses made with different combinations of CR/pepsin during 28 or 56 days of ripening.

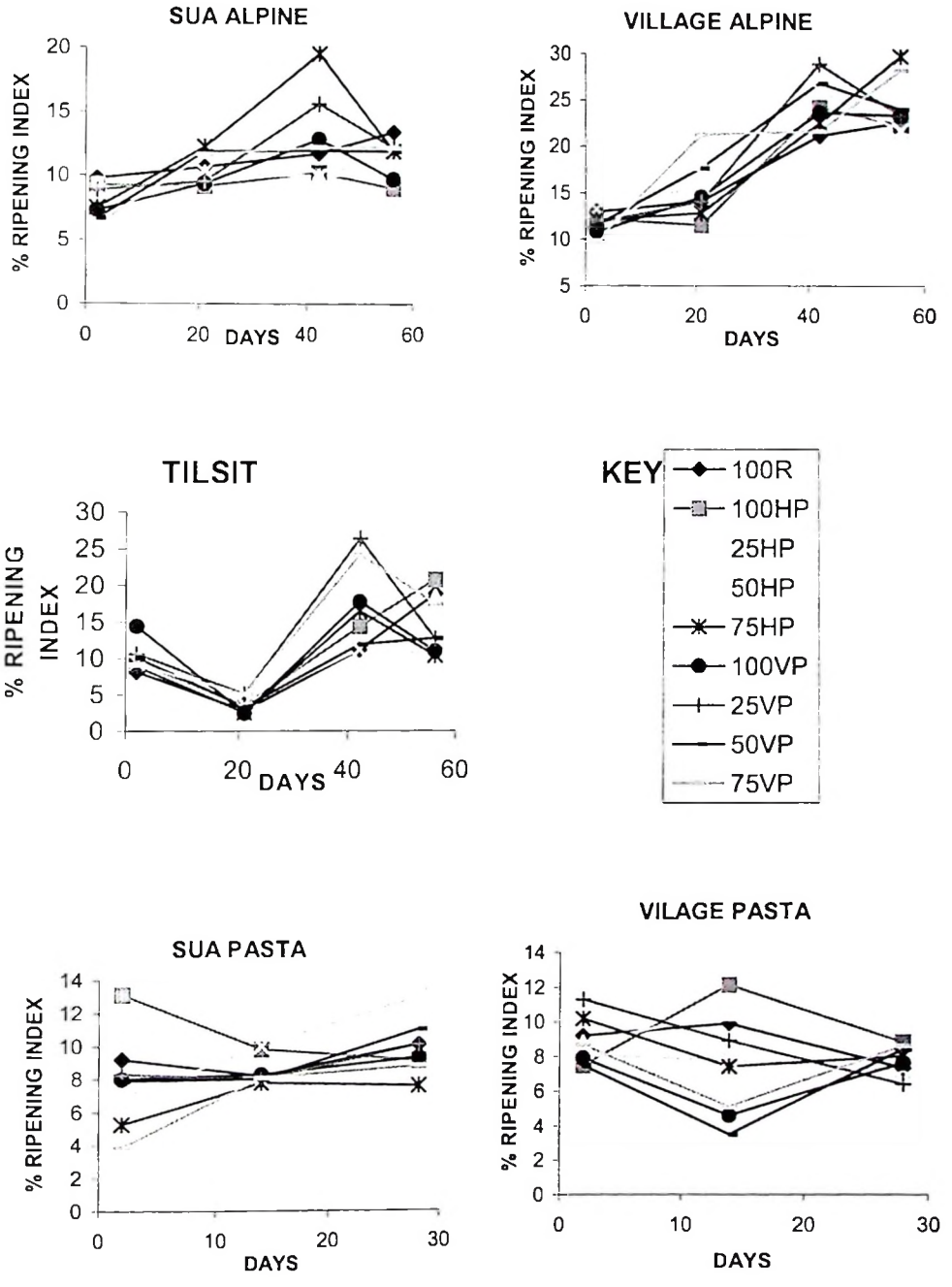
WSN in cheeses

The WSN for all types of cheese made with different enzyme combination are summarised in Table 15-19. WSN in all SUA Alpine cheeses increased with ripening time up to 42 days where WSN in some of them continued to increase up to day 56 while in others WSN decreased up to 56 days. Range at 42 days was 0.64% for 25HP to 1.17 % for 75 VP, and the control had 0.81%

In all village Alpine cheeses, WSN increased up to 42 days after which WSN in 75HP continued to increase up to day 56 while in all other cheese WSN decreased up to day 56. Range at 42 days was 1.64 % for 100HP to 1.85 % for 25VP and the control had 1.46 %. WSN in most Tilsiter cheeses increased up to 42 days except for 25HP in which WSN decreased up to 42 days after which WSN decreased in some cheese up to day 56 while in others it increased up to day 56. Range at 42 days was 0.53% for 25HP to 2.07% for 25 VP, and the control had 0.90%.

In most SUA Pasta cheeses WSN increased with ripening time up to 28 days except 100HP which had a higher WSN in the beginning which decreased up to 14 days and then levelled out. Range at 14 days was 0.54% for 100CR to 0.71% for 50HP.

WSN in most village Pasta cheeses decreased up to 14 days and then increased up to day 28 except for cheese 100HP and 25HP in which WSN increased up to 14 days. In all the cheese there was a general increase in WSN up to day 28. The range at 14 days was 0.35 % for 50VP to 1.07% for 100HP, and the control had 0.76 %.



Key: X axis = Percent water soluble nitrogen
Y axis = Days of cheese ripening

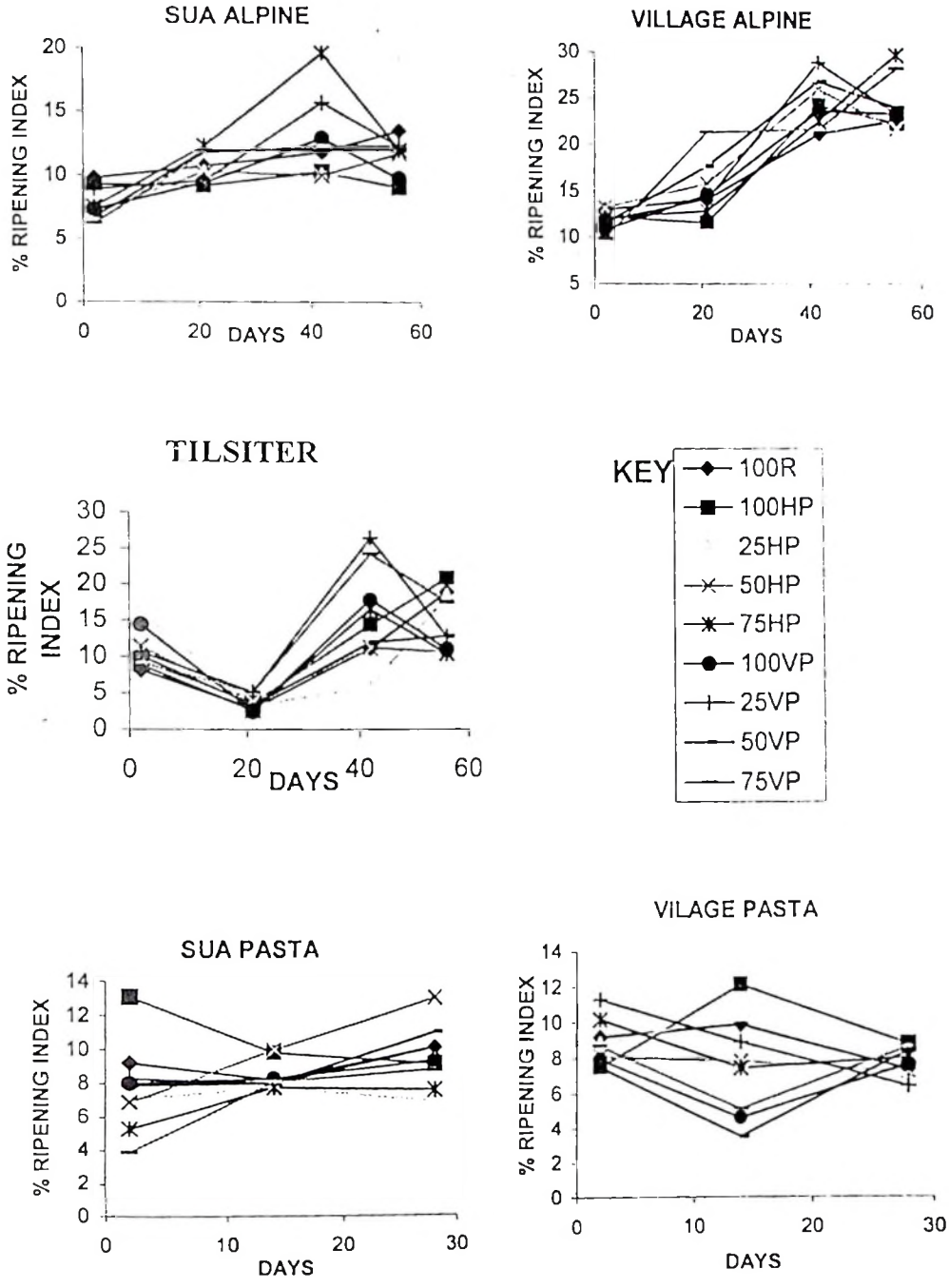
Figure 27: Water soluble nitrogen of cheeses made with different combinations CR/pepsin during 28 or 56 days of ripening

Ripening index

RI which is WSN percentage of TN for SUA Alpine cheeses, at 42 days was 9.9 for 50HP to 19.6% for 75HP and the control had 11.8% (Table 15-19)

For village Alpine the RI ranged from 20%for 100CR to 29% for 25VP.

RI for Tilsiter cheeses ranged from 6% for 25HP to 26% for 25VP and the control had 11%. For SUA Pasta the RI ranged from 7.8 for 75HP to 10% for 50HP and the control had 8%. RI for village Pasta cheeses ranged from 5% for 100VP to 12% for 100HP and the control had 10%.



Key: X axis = Percent ripening index
Y axis = Days of cheese ripening

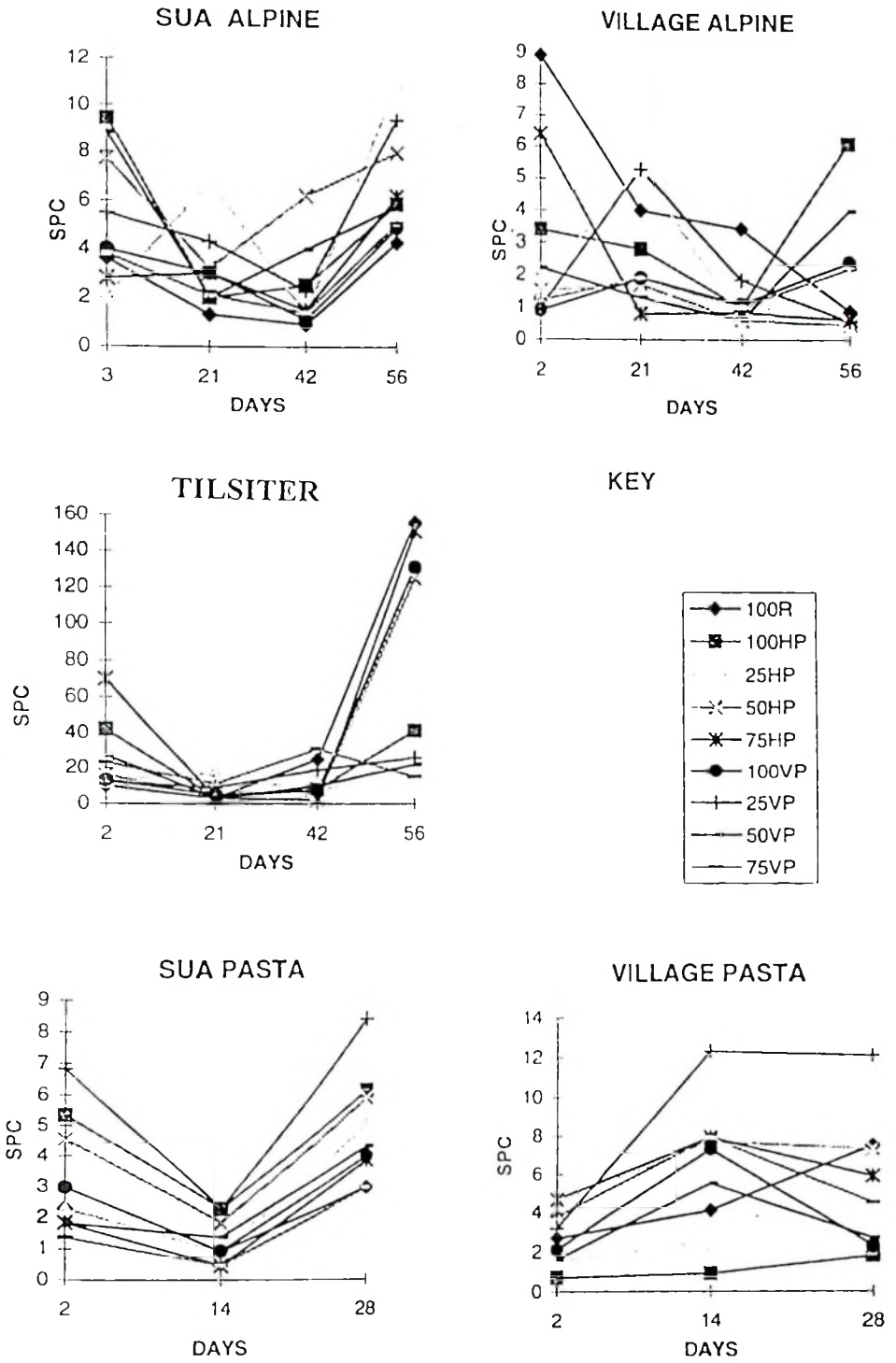
Figure 28: Ripening index of cheeses made with different combinations CR/pepsin during 28 or 56 days of ripening

4.3.3 Microbial quality of cheese

i) Standard plate count (SPC)

The trend of SPC for all types of cheese made with different enzyme combination are shown in Figure 28 and data at maturity are summarised in Table 15-19. SPC in SUA Alpine were high at day 2 in all the cheeses. However for the majority of cheeses SPC were greatly reduced by day 21 and even more by day 42 while the counts increased again up to the overipened stage at day 56. At 42 days the counts ranged from 0.9 to 6 x 10^5 cfu/g and the control had the lowest while 50HP had highest counts. In village Alpine cheeses, the SPC were initially high and then dropped with ripening time some at 21 days and most in 42 days, thereafter some increased and some decreased up to day 56. At 42 days the counts ranged from 0.6 for 50VP to 3 x 10^5 cfu/g and the control had the highest count.

Initially the SPC in Tilsiter cheeses were high then dropped up to 21 days. In few cheeses it increased slightly up to day 42 while most remained at the same level by day 42 after which counts increased up to day 56 for all cheese except for 75VP. Range at 42 days was 2 to 31 x 10^5 cfu/g while the control had 25 x 10^5 cfu/g. SPC in SUA Pasta were high by day 2, decreased up to day 14 and then increased again up to day 28. The trend of cheeses SPC count during ripening time was similar in all the cheese. Range at 14 days was 4 x 10^4 cfu/g for 75HP and 23 x 10^4 cfu/g for cheese 100HP and 25VP, and the control had 9 x 10^4 cfu/g. In village Pasta the SPC were low initially and then increased up to day 14 and then most cheeses had their counts reduced and some increase up to day 28. The range at day 14 was 0.9 x 10^4 cfu/g for 100HP to 12 x 10^4 cfu/g for cheese 25VP while the control had 4 x 10^4 cfu/g.



Key
X axis = SPC in SUA Alpine cheeses, Village Alpine and Tilsiter cheeses had counts of log 10^5 While SUA Pasta and village Pasta cheeses 10^4 cfu /g cheese
Y axis = Days of cheese ripening

Figure 29: SPC in cheese made with different combinations CR/pepsin during 28 or 56 days of ripening.

Coliform counts (coli)

The trend of coliform counts for all types of cheese made with different enzyme combination are shown in Figure 30, and data are summarised in Table 15-19.

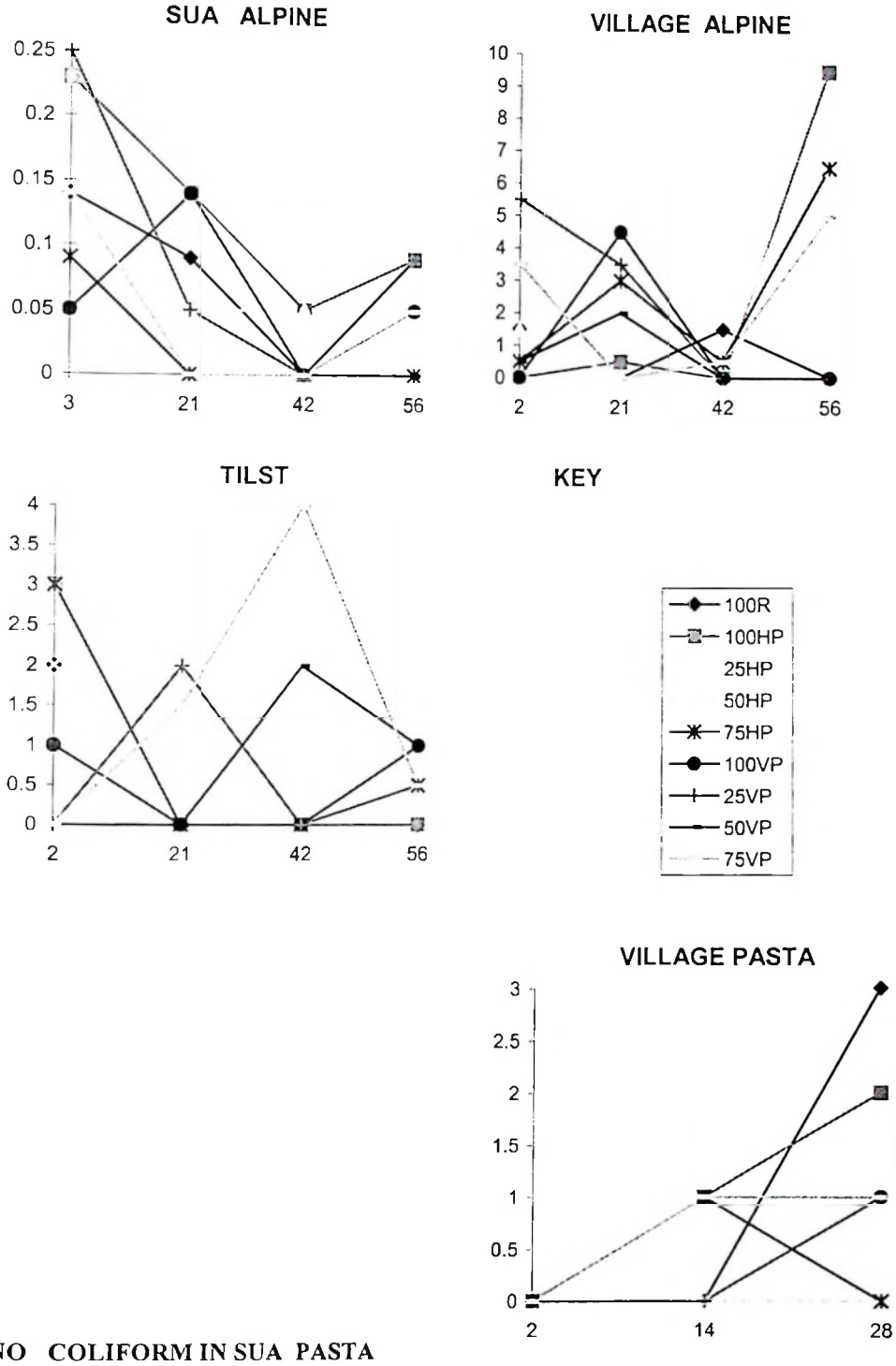
Overall, the coli counts in SUA Alpine cheeses decreased with ripening time, some decreased near to zero by day 21 or day 42. However, for some cheese the coli counts increased again up to day 56. Range by 42 days was $< 10^3$ cfu/g for most cheese to $1x\ 10^3$ cfu/g for 100HP and 25 HP.

In village Alpine cheeses, the coli counts at the beginning varied among cheeses, with some at $<10^3$ cfu/g, then some increased up to 21 days and all of them were reduced up to day 42 and thereafter some counts increased while others remained low at day 56. Range at 42 days was $< 10^3$ cfu/g for most cheese to 2×10^3 cfu/g for 100CR which was the control.

Coli counts in Tilsiter cheeses varied somewhat among cheeses with ripening time. The counts for several cheeses were reduced to $< 10^4$ cfu/g by day 21. Cheese 75VP counts increased from day 2 to 42 and were then reduced by day 56. Range at 42 days was $< 10^4$ cfu/g for all cheeses except 50VP and 75VP which had 2 and 4×10^4 cfu/g respectively.

No coliforms were observed for SUA Pasta cheeses.

The coli counts at start for Village Pasta cheeses were $< 10^4$ cfu/g. Thereafter coli were found in some cheeses and by 14 days the counts ranged from $< 10^4$ cfu/g for cheese 100CR, 25HP, 50 HP to 25VP and $1x10^3$ cfu/g in the remaining cheeses.



NO COLIFORM IN SUA PASTA

Key: X axis = Coliform counts in SUA Alpine cheeses, Village Alpine and Tilsiterer cheeses had counts of log 10^5 while SUA Pasta and village Pasta cheeses 10^4 cfu /g cheese
Y axis = Days of cheese ripening

Figure 30: Coliform counts in cheese with different combinations of CR/pepsin during 28 or 56 days of ripening.

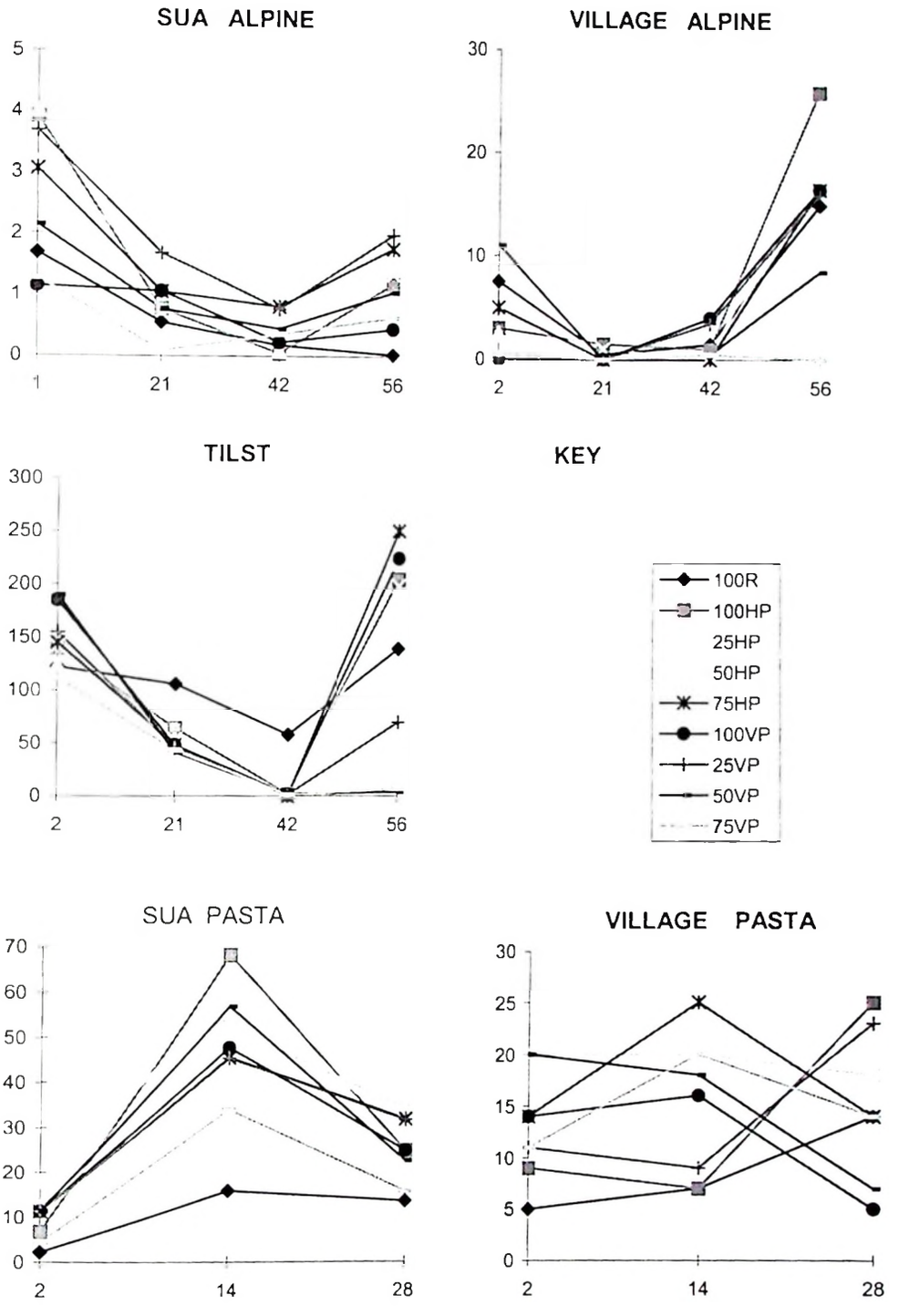
Yeast and mould count (YM)

The trend of YM for all types of cheese made with different enzyme combination are shown in Figure 31, and data are summarised in Table15-19.

YM in SUA Alpine cheeses were high in the beginning and were then reduced with ripening time up to 42 days after which they increased up to day 56. YM counts in cheese 75VP decreased up to 21 days then increased slightly up to day 42. Range of YM counts at 42 days was 0.4 x 10^5 cfu/g for 100CR to 4 x 10^5 cfu/g for 50HP.

Initially some village Alpine cheeses had YM counts $<10^4$ cfu/g and for others the counts up to 21days and thereafter YM counts in most cheeses increased up to day 56 except cheese 75VP with no counts $<10^4$ cfu/g. Range at 42 days was $<10^4$ cfu/g for cheese 25HP, 50HP, 75HP and 4 x 10^4 cfu/g for cheese 100VP and the control had 2 x 10^4 cfu/g. YM counts in all the Tilsiter cheeses decreased with ripening time up to 42 days, and thereafter the counts increased in all the cheeses except in cheese 75VP. At 42 days the range was $<10^5$ cfu/g for cheese 50HP, 75HP and 58 x 10^5 cfu/g in cheese 100CR.

In all the SUA Pasta cheeses, the YM counts decreased up to day 14 and then increased up to day 28. Range at 14 days was 2 x 10^4 cfu/g for cheese 100CR to 18 x 10^4 cfu/g for cheese 25 HP. YM counts in village Pasta cheeses varied differently for different cheese with counts decreasing in some and increasing in others up to 14 days and then increasing or decreasing in up to day 28 except for cheese 25HP and 100CR in which counts increased from day 2 to 28. Range at 14 days was 0.7 x 10^4 cfu/g for cheese 100CR, 100HP to 3 x 10^4 cfu/g for cheese 75HP.



Key
X axis = Yeast and mould counts (cfu /g) SUA Alpine 10^5 , village Alpine 10^4, Tilsiter 10^5 , SUA Pasta 10^4 and village Pasta 10^4. Y axis = Days of cheese ripening

Figure 31: Yeast and mould in cheeses with different combinations of CR/pepsin during 28 or 56 days of ripening

Table 15: LSmeans (± SE) of chemical composition and microbial quality of SUA Alpine cheese at 42 days of ripening

Variable	Rennet/ pepsin combinations								
	100R	100HP	25HP	50HP	75HP	100VP	25VP	50VP	75VP
PH	5.35 ±.001 [d]	5.58 ±.001 [a]	5.31 ±.001 [f]	5.32±.001 [c]	5.22 ±.001 [h]	5.25±.001 [g]	5.20 ±.001 [i]	5.36 ±.001 [c]	5.50±.001 [b]
Acid	3.50 ±.001 [b]	2.00±.001 [g]	3.65±.001 [a]	3.20±.001 [d]	2.85±.001 [e]	3.55±.001 [ab]	3.35±.001 [c]	2.65±.001 [f]	2.05±.001 [g]
MC	41.58±.03 [b]	40.07±.03 [c]	32.42±.03 [h]	33.93±.03 [f]	38.28±.03 [d]	40.11±.03 [c]	33.71±.03 [g]	37.93±.03 [e]	43.57±.03 [a]
BF	54.77±.28 [b]	53.39±.28 [c]	46.24±.28 [g]	49.19±.28 [f]	50.23±.28 [e]	51.76±.28 [d]	43.74±.28 [h]	54.77±.28 [b]	60.26±.28 [a]
Salt	4.54±.04 [b]	4.00±.04 [c]	2.88±.04 [ed]	2.72±.04 [f]	7.13±.04 [a]	4.42±.04 [b]	3.03±.04 [d]	1.28±.04 [g]	2.83±.04 [ef]
TN	6.87±.001 [c]	6.49±.001 [e]	6.42 ±.001 [g]	7.15±.001 [b]	5.98 ±.001 [I]	6.56 ±.001 [d]	6.26 ±.001 [h]	6.43 ±.001 [f]	7.86 ±.001 [a]
WSN	0.81± .11 [ab]	0.67 ± .11 [ab]	0.64 ± .11 [b]	0.71± .11 [ab]	1.17 ± .11 [a]	0.85± .11 [ab]	0.98 ± .11 [ab]	0.79 ± 11 [ab]	0.97 ± .11 [ab]
RI	11.8 ± 1.7 [ab]	10.3 ± 1.7 [b]	10.0 ± 1.7 [b]	9.9 ±1.7 [b]	19.56 ± 1.7 [a]	13.0 ±1.7 [ab]	15.7± 1.7 [ab]	12.3± 1.7 [ab]	12.3 ±1.7 [ab]
SPC[5]	0.9±.004 [a]	2.5± .004 [a]	1.3±.004 [a]	6.2± .004 [a]	1.5±.004 [a]	1.1± .004 [a]	2.3± .004 [a]	4.0± .004 [a]	1.4± .004 [a]
Coli[3]	0 ± .01 [a]	1±.01 [a]	1± .01 [a]	0± .01 [a]	0± .01 [a]	0±.01 [a]	0± .01 [a]	0± .01 [a]	0± .01 [a]
YM[5]	0.4± ..003 [a]	1.2 ± .003 [a]	2 ± .003 [a]	4± .003 [a]	1.8± .003 [a]	0.5± .003 [a]	1.7± .003 [a]	1± .003 [a]	0.8± .003 [a]

Within the row, means followed by the same superscript do not differ significantly at P>0.05 according to DMRT

Key of Tables

BF = butterfat

MC= moisture

TN = total nitrogen

WSN= water soluble nitrogen

RI= ripening index DRMT = Duncan's Multiple Range Test

Superscripts on SPC, coli and YM represents the counts in cfu/g in cheese

Table 16: LSmeans (± SE) of chemical composition and microbial quality of village Alpine cheese at 42 days of ripening.

Variable	Rennet/ pepsin combinations								
	100R	100HP	25HP	50HP	75HP	100VP	25VP	50VP	75VP
PH	5.96±.001 ^{d}	5.90 ± .001 ^{e}	6.36± .001 ^{a}	5.86 ± .001 ^{f}	5.85 ± .001 ^{f}	6.01 ± .001 ^{c}	6.01 ± .001 ^{c}	6.03± .001 ^{b}	6.04±.001 ^{b}
Acid	2.2± .001 ^{f}	3.3± .001 ^{e}	3.2± .001 ^{e}	3.4± .001 ^{a}	3.8± .001 ^{d}	3.4±.001 ^{a}	3.5±.001 ^{cd}	3.6±.001 ^{cb}	3.6±.001 ^{b}
MC	34.9±.18 ^{b}	35.89 ±.18 ^{a}	32.34 ±.18 ^{e}	33.87 ±.18 ^{c}	33.15 ±.18 ^{d}	34.39 ±.18 ^{bc}	34.35 ±.18 ^{bc}	34.65 ±.18 ^{b}	32.05 ±.18 ^{e}
BF	67.60±.82 ^{a}	53.06 ±.82 ^{bc}	52.47 ±.82 ^{bc}	54.44 ±.82 ^{b}	54.60 ±.82 ^{b}	53.72 ±.82 ^{b}	50.65 ±.82 ^{c}	54.33 ±.82 ^{b}	45.25±.82 ^{d}
Salt	1.61±.01 ^{g}	2.35±.01 ^{d}	2.73±.01 ^{b}	4.29±.01 ^{a}	4.27±.01 ^{a}	2.67±.01 ^{c}	1.90±.01 ^{f}	1.99±.01 ^{e}	1.91±.01 ^{f}
TN	7.25±.15 ^{a}	6.97±.15 ^{abc}	6.34±.15 ^{d}	6.78±.15 ^{abcd}	6.74±.15 ^{bcd}	6.51±.15 ^{bcd}	6.48±.15 ^{cd}	7.01±.15 ^{ab}	6.60±.15 ^{bcd}
WSN	1.46±.03 ^{ed}	1.64 ± .03 ^{g}	1.58 ±.03 ^{c}	1.64 ±.03 ^{d}	1.52 ±.03 ^{gf}	1.72 ±.03 ^{ef}	1.85 ±.03 ^{gf}	1.71 ±.03 ^{b}	1.43 ±.03 ^{a}
RI	20.0±.7 ^{ed}	24.0 ±.7 ^{f}	25.0 ±.7 ^{b}	24 ±.7 ^{c}	23 ±.7 ^{ef}	26 ±.7 ^{cd}	29 ±.7 ^{de}	24 ±.7 ^{b}	22 ±.7 ^{a}
SPC[5]	3.4±.008 ^{ab}	1.0 ±.008 ^{ab}	0.5 ± .008 ^{ab}	0.5 ±.008 ^{ab}	0.8 ± .008 ^{a}	1.1± .008 ^{ab}	1.8±.008 ^{ab}	0.6±.008 ^{b}	1.0± .008 ^{ab}
Coli[3]	1.5± .03 ^{ab}	0 ± .03 ^{ab}	0± .03 ^{ab}	0.5±.03 ^{ab}	0± .03 ^{a}	0± .03 ^{ab}	0± .03 ^{ab}	0± .03 ^{ab}	0.5±.03 ^{ab}
YM[4]	1.5±.031 ^{bc}	1± .031 ^{c}	0±.031 ^{c}	0±.031 ^{c}	0±.031 ^{c}	4±.031 ^{a}	3.5± .031 ^{ab}	0.5±.031 ^{c}	0.5±.03 1 ^{c}

Within the row, means followed by the same superscript do not differ significantly at P>0.05 according to DMRT

Key of Tables

BF = butterfat MC= moisture

TN = total nitrogen WSN= water soluble nitrogen

RI= ripening index DRMT = Duncan's Multiple Range Test

Superscripts on SPC, coli and YM represents the counts in cfu/g in cheese

Table 17: LSmeans (± SE) of chemical composition and microbial quality of Tilsiter cheese at 42 days of ripening

Variable	Rennet/ pepsin combinations								
	100R	100HP	25HP	50HP	75HP	100VP	25VP	50VP	75VP
pH	5.51±.01 [b]	5.34 ±.01 [d]	5.02 ±.01 [g]	5.0 ±.01 [g]	5.03 ±.01 [e]	5.63 ±.01 [c]	5.51 ±.01 [b]	5.12 ±.01 [f]	5.61 ±.01 [a]
Acid	3.65±.01 [cd]	3.75±.01 [cd]	2.65±.01 [f]	4.95±.01 [a]	3.85±.01 [e]	4.75±.01 [b]	4.20±.01 [b]	3.05±.01 [e]	3.60±.01 [d]
MC	32.66±.18 [b]	30.14±.18 [c]	28.34±.18 [e]	25.87±.18 [g]	27.37±.18 [f]	29.27±.18 [d]	38.28±.18 [a]	27.77±.18 [ef]	32.81±.18 [b]
BF	50.12 ±.61 [cb]	48.67±.61 [cd]	52.33±.61 [a]	47.89±.61 [de]	51.63±.61 [ab]	43.12±.61 [f]	52.65±.61 [a]	46.38±.61 [e]	46.51±.61 [e]
Salt	0.74±.02 [a]	1.71±.02 [g]	2.58±.02 [c]	3.16±.02 [b]	2.06±.02 [e]	4.09±.02 [a]	2.43±.02 [d]	1.94±.02 [f]	1.79±.02 [g]
TN	8.03±.16 [c]	6.82 ±.02 [f]	9.07 ±.02 [a]	8.32±.02 [b]	8.06±.02 [c]	8.04±.02 [c]	7.83±.02 [d]	8.06±.02 [c]	6.96±.02 [e]
WSN	0.90 ±.03 [f]	0.98 ±.03 [e]	0.53 ±.03 [g]	0.93 ±.03 [ef]	1.33 ±.03 [d]	1.44 ±.03 [c]	2.07 ±.03 [a]	0.96±.03 [ef]	1.68±.03 [b]
RI	11±.35 [f]	14±.35 [e]	6±.35 [g]	11±.35 [f]	17±.35 [d]	17±.35 [d]	26 ±.35 [a]	12±.35 [f]	24±.35 [b]
SPC[5]	25±.003 [a]	8± .003 [c]	6±.003 [d]	4± .003 [e]	2± .003 [f]	7± .003 [d]	19±.003 [b]	10± .003 [c]	31±.003 [a]
Coli[4]	0±.002 [a]	0 ±.002 [a]	0 ± .002 [a]	0 ± .002 [a]	0 ±2.002 [a]	0 ± .002 [a]	0 ± .002 [a]	2 ± .002 [b]	4 ± .002 [c]
YM[5]	58±.002 [b]	2 ± .002 [b]	1±002 [a]	0±.002 [a]	0±.002 [a]	1±002 [a]	1± .002 [a]	2± .002 [b]	1± .002 [a]

Within the row, means followed by the same superscript do not differ significantly at P>0.05 according to DMRT

Key of Tables

BF = butterfat

MC= moisture

TN = total nitrogen

WSN= water soluble nitrogen

RI= ripening index DRMT = Duncan's Multiple Range Test

Superscripts on SPC, coli and YM represents the counts in cfu/g in cheese

Table 18: LSmeans (± SE) of chemical composition and microbial quality of SUA Pasta cheese at 14 days of ripening

Variable	Rennet/ pepsin combinations								
	100R	100HP	25HP	50HP	75HP	100VP	25VP	50VP	75VP
PH	5.16±.01^{c}	5.43 ±.01^{b}	5.16 ±.01^{c}	5.11 ±.01^{d}	5.44 ±.01^{b}	5.46 ±.01^{a}	5.09 ±.01^{e}	5.12 ±.01^{d}	5.08 ±.01^{e}
Acid	2.85±.01^{b}	2.6±.01^{c}	2.20±.01^{d}	3.00±.01^{ab}	1.60±.01^{e}	2.65±.01^{c}	2.30±.01^{d}	2.65±.01^{c}	3.05±.01^{a}
MC	44.05±.02^{f}	46.36±.02^{b}	44.99±.02^{d}	49.09±.02^{a}	43.70±.02^{g}	44.34±.02^{d}	42.30±.02^{h}	43.65±.02^{g}	45.29±.02^{c}
BF	44.20 ±.79^{b}	38.22±.79^{c}	45.45±.79^{b}	48.13±.79^{a}	43.52±.79^{b}	32.79±.79^{d}	43.76±.79^{b}	45.24±.79^{b}	45.69±.79^{ab}
Salt	2.59±.08^{e}	5.87±.08^{a}	4.18±.08^{c}	1.47±.08^{g}	1.51±.08^{g}	4.85±.08^{b}	3.98±.08^{c}	2.31±.08^{f}	2.93±.08^{d}
TN	6.61±.11^{e}	6.51 ±.11^{f}	7.40±.11^{b}	7.07±.11 d	7.23±.11^{c}	7.05±.11^{d}	7.07±.11^{d}	7.45±.11^{a}	7.40±.11^{b}
WSN	0.54 ±.02^{c}	0.64 ±.02^{b}	0.58 ±.02^{cb}	0.71 ±.02^{a}	0.57 ±.20^{c}	0.59 ±.02^{cb}	0.57 ±.02^{c}	0.60±.02^{cb}	.60 ±.02^{cb}
RI	8.2 ±.27^{b}	9.8±.27^{a}	7.8 ±.27^{c}	10 ±.27^{a}	7.8±.27^{c}	8.3±.27^{b}	8.0±.03^{b}	7.9±.27^{b}	8.1±.27^{b}
SPC[4]	9.1±.001^{a}	22.7±.001^{a}	9.0±.001^{a}	18.9±.001^{a}	4.5±.001^{a}	9.0±.001^{a}	22.9±.001^{a}	13.6±.001^{a}	4.5±.001^{a}
Coli[0]	0±.01^{a}	0 ±.01^{a}	0 ±.01^{a}	0 ±.01^{a}	0 ±.01^{a}	0 ±.01^{a}	0 ±.01^{a}	0 ±.01^{a}	0±.01^{a}
YM[4]	2.2±.01^{a}	6.8 ±.01^{a}	18.1±.01^{a}	13.6±.01^{a}	11.3±.01^{a}	11.3±.01^{a}	11.3±.01^{a}	11.4±.01^{a}	4.5±.01^{a}

Within the row, means followed by the same superscript do not differ significantly at P>0.05 according to DMRT

Key of Tables

BF = butterfat MC= moisture

TN = total nitrogen

WSN= water soluble nitrogen

RI= ripening index DRMT = Duncan's Multiple Range Test

Superscripts on SPC, coli and YM represents the counts in cfu/g in cheese

Table 19: LSmeans (± SE) of chemical composition and microbial quality of village Pasta cheese at 14 days of ripening

Variable	Rennet/ pepsin combinations								
	100R	100HP	25HP	50HP	75HP	100VP	25VP	50VP	75VP
PH	4.83±.00^{d}	4.80±.00^{e}	4.90 ±.00^{a}	4.77±.00^{f}	4.78±.00^{f}	4.72±.00^{g}	4.88±.00^{b}	4.80±.00^{d}	4.86±.00^{c}
Acid	4.0 ±.00^{b}	4.4 ±.00^{a}	3.9±.00^{c}	3.5 ±.00^{d}	3.5 ±.00^{d}	3.2 ±.00^{e}	3.2 ±.00^{e}	3.0 ±.00^{f}	2.7 ±.00^{g}
MC	40.36±.04^{e}	44.34 ±.04^{a}	41.43 ±.04^{c}	40.71 ±.04^{d}	36.66 ±.04^{h}	39.97 ±.04^{g}	40.20 ±.04^{f}	41.38 ±.04^{c}	41.59 ±.04^{b}
BF	36.47±.59^{cd}	44.47±.59^{a}	40.12 ±.59^{b}	40.90 ±.59^{b}	36.31 ±.59^{cd}	35.40 ±.59^{d}	41.80 ±.59^{b}	36.25 ±.59^{cd}	39.67 ±.59^{c}
Salt	5.53±.08^{a}	4.76 ±.08^{b}	3.33 ±.08^{d}	3.37 ±.08^{d}	2.05 ±.08^{f}	1.75 ±.08^{f}	3.76 ±.08^{c}	2.30 ±.08^{e}	1.20 ±.08^{g}
TN	7.59±.01^{f}	8.87 ±.01^{a}	8.25±.01^{d}	8.34±.01^{c}	7.14±.01^{h}	7.73±.01^{e}	7.40±.01^{g}	8.44±.01^{b}	8.26±.01^{d}
WSN	0.76±.02^{c}	1.07 ±.02^{a}	0.87 ±.02^{b}	0.65 ±.02^{e}	0.53 ±.02^{g}	0.35 ±.02^{g}	0.69 ±.02^{d}	0.66 ±.02^{h}	0.42 ±.02^{f}
RI	10.0±.23^{d}	12.0±.23^{a}	11.0±.23^{b}	8.0±.23^{e}	7.0±.23^{c}	5±.23^{d}	9±.23^{b}	8±.23^{e}	5±.23^{d}
SPC[4]	4.1± .004^{a}	0.9 ±.00 4^{a}	2.5 ± .004^{a}	7.7 ± .004^{a}	7.9 ± .004^{a}	7.3 ± .004^{a}	12.3 ± .004^{a}	5.5 ± .004^{a}	7.9 ± .004^{a}
Coli[3]	0±.02^{a}	1±.02^{a}	0±.02^{a}	0±.02^{a}	1±.02^{a}	1±.02^{a}	0±.02^{a}	1±.02^{a}	1±.02^{a}
YM[4]	0.7±.001^{a}	0.7±.001^{a}	2.3±.001^{a}	2 ± .001^{a}	2.5 ± .001^{a}	1.6 ±.001^{a}	0.9 ±.001^{a}	1.8 ±.001^{a}	2 ±.001^{a}

Within the row, means followed by the same superscript do not differ significantly at P>0.05 according to DMRT

Key of Tables

BF = butterfat

MC= moisture

TN = total nitrogen

WSN= water soluble nitrogen

RI= ripening index

DRMT = Duncan's Multiple Range Test

Superscripts on SPC, coli and YM represents the counts in cfu/g in cheese

4.3.4 Sensory evaluation of cheese

Sensory evaluation of cheeses was treated by one way analysis of variance using the Bonferroni test.

Cheese taste

The trends of cheese taste for all types of cheese made with different enzyme combinations are summarised in Table 20.

The taste in SUA Alpine cheese 100CR, 100VP, 50VP were not significantly different from each other (P>0.05) while cheese 25HP Vs 100VP, 50VP Vs 25HP, 50HP Vs 100 VP were significant different (P<0.05) and cheese 100VP Vs 75VP, 50VP Vs 75VP were highly significantly different (P<0.01). Village Alpine, Tilsiter, SUA Pasta and village Pasta, were not significantly different in taste (P>0.05)

Table 20: Mean squares for the taste scores of cheeses.

Cheese	N	100 CR	100 HP	25 HP	50HP	75HP	100 VP	25VP	50VP	75VP
SUA Alpine	17	1.9± 0.13^{c}	2.1± 0.13^{a}	2.5± 0.15^{b}	2.5± 0.12^{b}	2.4± 0.12^{b}	1.8± 0.13^{c}	2.1± 0.16^{a}	1.8± 0.15^{c}	2.7± 0.15^{b}
Village Alpine	18	1.6± 0.14^{a}	1.8± 0.13^{a}	1.7± 0.14^{a}	2.0± 0.14^{a}	2.2± 0.17^{a}	1.9± 0.20^{a}	2.1± 0.15^{a}	1.6± 0.14^{a}	1.9± 0.15^{a}
Tilsiter	19	2.0± 0.15^{a}	2.1± 0.14^{a}	2.3± 0.17^{a}	2.1± 0.17^{a}	2.1± 0.15^{a}	1.9± 0.15^{a}	1.8± 0.16^{a}	2.0± 0.13^{a}	1.8± 0.18^{a}
SUA Pasta	15	1.9± 0.15^{a}	2.1± 0.15^{a}	2.1± 0.2^{a}	2.0± 0.17^{a}	1.9± 0.18^{a}	1.7± 0.16^{a}	1.5± 0.13^{a}	1.9± 0.23^{a}	1.7± 0.18^{a}
Village Pasta	16	1.8± 0.10^{a}	2.1± 0.17^{a}	2.2± 0.19^{a}	2.2± 0.19^{a}	2.1± 0.17^{a}	2.1± 0.17^{a}	2.2± 0.21^{a}	2.1± 0.15^{a}	2.2± 0.23^{a}

Cheese with the similar letter in the same row are not significantly different (P>0.05) according to DMRT

Cheese smell

The data for cheese smell for all types of cheese made with different enzyme combination are summarised in Table 21.

None of the cheeses were significantly different (P>0.05) in smell.

Table 21: Mean squares for smell scores of cheeses

Cheese	N	100 CR	100 HP	25 HP	50HP	75HP	100 VP	25VP	50VP	75VP
SUA Alpine	17	1.9± 0.15[a]	2.0± 0.15[a]	2.1± 0.17[a]	1.9± 0.16[a]	2.1± 0.18[a]	1.8± 0.16[a]	1.9± 0.17[a]	1.8± 0.15[a]	2.1± 0.17[a]
Village Alpine	18	1.7± 0.14[a]	2.0± 0.08[a]	1.9± 0.15[a]	2.1± 0.14[a]	1.8± 0.15[a]	1.9± 0.17[a]	2.0± 0.14[a]	1.9± 0.16[a]	1.9± 0.16[a]
Tilsiter	19	1.9± 0.15[a]	2.0± 0.12[a]	1.6± 0.16[a]	1.7± 0.13[a]	2.0± 0.15[a]	1.9± 0.15[a]	2.0± 0.15[a]	1.6± 0.14[a]	1.8± 0.17[a]
SUA Pasta	15	1.7± 0.15[a]	1.8± 0.14[a]	2.1± 0.15[a]	1.9± 0.15[a]	1.9± 0.15[a]	1.9± 0.17[a]	1.7± 0.16[a]	1.9± 0.18[a]	1.9± 0.15[a]
Village Pasta	16	1.7± 0.15[a]	1.7± 0.15[a]	1.9± 0.14[a]	1.9± 0.11[a]	1.9± 0.17[a]	1.9± 0.14[a]	1.9± 0.17[a]	2.2± 0.19[a]	2.3± 0.18[a]

Cheese with the similar letter in the same row are not significantly different (P>0.05) according to DMRT

Cheese appearance

The trends of cheese appearance for all types of cheese made with different enzyme combinations are summarised in Table 22.

The appearance of SUA Alpine, Tilsiter and village Pasta cheeses were not significantly different (P>0.05) except that village Alpine cheese 100CR vs 75HP and SUA Pasta cheese 100CR vs 50HP were significantly different (P<0.05)

Table 22: Mean squares for appearance scores of cheeses

Cheese	N	100 CR	100 HP	25 HP	50HP	75HP	100 VP	25VP	50VP	75VP
SUA Alpine	17	1.9± 0.21[a]	1.7± 0.19[a]	2.2± 0.18[a]	2.4± 0.15[a]	1.8± 0.20[a]	1.8± 0.14[a]	2.3± 0.19[a]	2.1± 0.17[a]	2.3± 0.14[a]
Village Alpine	18	1.5± 0.15[a]	2.1± 0.15[a]	1.7± 0.16[a]	2.2± 0.12[a]	2.3± 0.14[a]	1.9± 0.17[a]	2.2± 0.17[a]	1.7± 0.11[a]	1.9± 0.16[a]
Tilsiter	19	1.6± 0.14[a]	2.1± 0.14[a]	2.0± 0.19[a]	1.9± 0.15[a]	2.2± 0.18[a]	2.2± 0.14[a]	1.9± 0.18[a]	2.0± 0.17[a]	2.1± 0.20[a]
SUA Pasta	15	1.4± 0.16[a]	2.1± 0.18[b]	2.2± 0.20[b]	2.3± 0.16[c]	2.1± 0.15[b]	1.7± 0.20[b]	1.5± 0.17[a]	1.9± 0.17[b]	1.7± 0.16[b]
Village Pasta	16	1.9± 0.21[a]	1.9± 0.15[a]	2.1± 0.14[a]	1.8± 0.16[a]	1.8± 0.14[a]	1.8± 0.17[a]	2.1± 0.17[a]	1.8± 0.11[a]	1.9± 0.19[a]

Cheese with the similar letter in the same row are not significantly different (P>0.05) according to DMRT

Bitterness in cheese

The data for cheese bitterness for all types of cheese made with different enzyme combination are summarised in Table 23.

SUA Alpine cheese varied significantly in bitterness: cheese 50HP differed from 100CR, 100HP, 100VP and cheese 25VP differed from 75VP, 75HP at P<0.05) while cheese 100CR differed much from 75HP, 75VP and cheese 25HP differed much from 50VP (P<0.01). Cheese 25HP differed very much from 100CR, 100VP, 100HP, 25 VP while 100VP differed very much from 75HP, 75VP (P<0.0001) Village Alpine and Village Pasta cheese were not significantly different in bitterness (P>0.05).

Tilsiter cheeses were not significantly different in bitterness (P>0.05) except that 100CR versus (vs) 50HP, 100HP vs 75, 50VP vs 25VP and 75VP vs 25VP were significantly different (P<0.05)

SUA Pasta cheese were not significantly different in bitterness (P>0.05) except that 25HP was significantly different from 25VP (P<0.05)

Table 23: Mean Squares for bitterness scores of cheeses

Cheese	N	100 CR	100 HP	25 HP	50HP	75HP	100 VP	25VP	50VP	75VP
SUA Alpine	17	1.5± 0.15^{a}	1.5± 0.15^{a}	2.7± 0.1^{b}	2.2± 0.16^{c}	2.5± 0.15^{d}	1.4± 0.12^{a}	1.7± 0.1^{e}	1.8± 0.18^{c}	2.5± 0.12^{a}
Village Alpine	18	1.6± 0.16^{a}	1.6± 0.14^{a}	1.7± 0.18^{a}	1.9± 0.16^{a}	1.9± 0.20^{a}	2.1± 0.21^{a}	2.1± 0.16^{a}	1.7± 0.14^{a}	1.7± 0.18^{a}
Tilsiter	19	1.2± 0.09^{a}	1.4± 0.14^{a}	1.2± 0.12^{a}	1.8± 0.18^{b}	1.8± 0.16^{b}	1.7± 0.12^{b}	1.1± 0.07^{a}	1.4± 0.11^{a}	1.5± 0.16^{c}
SUA Pasta	15	1.5± 0.19^{a}	1.7± 0.16^{b}	2.0± 0.14^{b}	1.7± 0.15^{b}	1.5± 0.13^{a}	1.5± 0.17^{a}	1.1± 0.08^{c}	1.7± 0.21^{b}	1.5± 0.13^{a}
Village Pasta	16	1.6± 0.16^{a}	1.7± 0.18^{a}	1.4± 0.15^{a}	1.6± 0.15^{a}	1.4± 0.13^{a}	1.5± 0.17^{a}	1.7± 0.22^{a}	1.5± 0.18^{a}	1.4± 0.18^{a}

Cheese with the similar letter in the same row are not significant different (P>0.05) according to DMRT

CHAPTER 5

DISCUSSION

5.1 Experiment 1: Extraction of bovine pepsin

5.1.1 Optimisation of extraction conditions for maximum MCA of crude extract

Pepsin concentration in the fundic vs pyloric region of abomasa

This study showed that pepsin concentration in the fundic was significantly higher than in the pyloric regions (P>0.001). Similar findings have been reported by Hagyard and Davey (1972); Garnot *et al.* (1977); Andren *et al.* (1981) who reported that pepsin concentration was strongly influenced by age, feeding regime, and individual ability to produce the enzyme. The total pepsin concentration of 1.08 mg/ml and 0.46 mg/ml in the extracts from the fundic and pyloric regions respectively were within the range reported by Andren *et al.* (1981) who found 1-2 mg/ml. The pepsin concentration in fundic region was 2.4 to 2.6 times higher than in the pyloric region. Andren *et al.* (1981) reported that the differences of bovine pepsin in the two regions was obvious due to the anatomical histological differences existing in the abomasum. The pyloric region mostly contains mucin-producing cells while the fundic region contains proportionally more enzyme-producing cells. Therefore the fundic region should be used for pepsin extraction and the cost may be reduced by buying the fundic region only. However, this is difficult unless the current marketing system is changed whereby the fundic region could be sold separately.

Pepsin from dried versus fresh abomasa

Dried abomasa gave higher MCA than fresh abomasa. Similar results on extraction of dry versus fresh abomasa was reported by Hewedy *et al.* (1992) who obtained higher MCA in milled dried or sliced dried tissue than fresh minced calf tissue. They reported that drying modifies the mucus nature of the abomasa which then resulted in high MCA of extracts. Also Hagyard and Davey (1974) showed that dried veils increased the yield of MCA by 11% over that obtained from fresh veil materials. The same trend of results was obtained in the present study where yield of MCA was 29.4% higher in dried compared to extracts from fresh fundic region of the abomasa. It can be concluded that drying should be preferred as it is an appropriate and cheap technology for Tanzania.

Effect of drying methods

The higher MCA in extract from sun dried abomasa was also reported by Hagyard Davey (1974) who found out that sun-shine or sun rays reduce the viscous or mucus nature of the abomasa tissue and the ratio of the various zymogens present in the pro-chymosin and pepsinogen so that during the enzyme extraction process there is an increase in yield and a favoured conversion of prochymosin or pepsinogen

The effect of direct sun drying on the abomasa also explains why the dry matter percentage in the sun dried abomasa extract was lower than extract from abomasa dried under shade. This could be because the un-modified mucus decomposed during long drying period of the abomasa under the shade and were included in the extracts during pepsin extraction hence increasing the dry matter.

The microbial quality showed no significant difference ($P>0.05$). However, the calculated mean values of 2.3×10^4 cfu/ml of SPC was lower than that for shade dried extract which was 2.7×10^4 cfu/ml implying better microbial quality of crude extract obtained by sun drying. The study agree with the report by Hagyard and Davey (1974) who indicated that sundrying improves microbial quality of crude extract and preserves the enzyme. That is why there was low microbial counts in sun dried extracts in the present study while long drying periods under the shade favoured increase in bacterial counts.

Effect of ionic strength of extraction solution

In the present study, there was a direct relationship between NaCl concentration and MCA of the extract up to 10 % NaCl. Concentration of more than 10% NaCl had a negative effect on MCA and this is in agreement with findings of Whitaker (1972); O'Leary and Fox (1975); Visiteva and Isyrenova (1985) and Lyatuu (1991) who reported that yield and MCA increased by increasing NaCl concentration between 5-10 % NaCl. Whitaker (1972) reported that low and high NaCl concentration would normally reduce the ability of the extraction solution to de-sorb the enzyme from its attachments. Moreover, Anis *et al.* (1983) reported that the direct relationship between NaCl concentration and MCA was due to the phenomena and theory of partial NaCling out of the enzyme as well as the cation exchange taking place between added NaCl and calcium ions of milk which results in extended clotting time.

Effect of optimum extraction temperature

Herrian *et al.* (1983) noted that the 37°C, the temperature in the stomach of the cow, is in favour of crude pepsin activation because the conversion of inactive pepsinogen to active pepsin take place under normal physiological conditions. This observation was in agreement with the present findings where the highest MCA was obtained with extraction at 37°C followed by room temperature. In other studies elsewhere, optimum pepsin extraction has been reported at various temperatures 37-42°C by Fox (1969); Nelson (1975); 28- 32°C by Davide *et al.* (1982); Peralta *et al.* (1986); 40-42° by Herrian *et al.* (1983); 26°C by Visiteva and Isyrenova (1985). The high temperatures used in these studies normally required available electricity and maintenance of incubators for pepsin maceration. Under Tanzanian conditions, however, extraction at 25-30°C is most appropriate because it is the temperature found in most parts of the country. Small scale cheese processors will not need to use incubators but would take advantage of the prevailing high ambient temperatures throughout the year to extract bovine pepsin. In colleges, universities and processing plants where incubators are available, 37 °C could be recommended for extraction.

Effect of extraction time

From the present study, optimum MCA (Figure 7) was obtained when the dried abomasa were macerated for 3 days at pH 3. This period was found appropriate because on the 4th day there was not much increase of activity except some in the 5th day, showing that 3 days was optimum period for extraction. This result disagree with findings by Hewedy

et al. (1992) who reported that MCA of calves and adult cattle abomasa had a gradual increase in activity up to 8 days then decreased on the 10 th day. The differences in findings between the present study and those of Hewedy *et al.* (1992) could be due to variations in temperatures, pH, NaCl content and the preservative they used in their extraction. The increase in activity in their study could not be explained clearly, but Nielsen and Foltmann (1993) found that crude preparations of pepsin could contain appreciable amounts of the dissociable pepsin inhibitors liberated during activation of pepsinogen. As the time passes, these inhibitors keep on dissociating and increasing the proportion of the enzyme in the extract. Furthermore, abomasal preparations privide conducive conditions for acid tolerant organisms which may encourage milk coagulation or due to more acidic environment created by their metabolites may lower the pH of the preparation and increase the MCA (Fox *et al.*1995). However, acid tolerant organisms or dissociable pepsin inhibitors were not measured in the present study. Therefore a period of three days was recommended for pepsin extraction.

Effect of activation pH and extraction time

The results of activation at pH 2 was in agreement with the findings of Foltmann, (1960); Harboe *et al.* (1974); Nielsen and Foltmann (1993) who reported that activation of zymogens was fastest at pH 2 where pepsin has its optimal proteolytic activity, and pH 2 has been taken as a measure for the conversion of pepsinogen into pepsin. Results (Figure 8) show that conversion of pepsinogen to pepsin increased as pH decreased with highest MCA at pH 1, but the enzyme obtained at this pH was not stable. This was in

agreement with the findings of Rajaopalan, *et al.* (1966) who indicated that it was due to the formation of active species when activation pH was low. The curve at pH 2 was very stable (Plateau-like shape) at 30 to 60 min showing presumably that all the pepsinogen had been converted to pepsin. Similar results were reported by Harboe *et al.* (1974) who found out that when pepsinogen is activated at pH 2 for a minimum time of 20 min it achieves full conversion to pepsin.

Furthermore Foltmann (1960) found that activation was accompanied by hydrolysis of a peptide bond near the NH_2-terminal end of the molecule. Enzyme activity increased with time after activation and Foltmann (1993) revealed the non-covalent intermediates between prosegment of peptides and pepsin and isolated complexes of Leu1p-Leu44p/pepsin and Leu1p-Leu16p/ pepsin. This agrees with the present study in which MCA increased after activation at different pH, but prosegment peptides were not isolated therefore further studies in this area is required.

The short time of activation for 30 min is important in that the small scale cheese processors (SSCP) will not wait for a long time for the activation of the enzyme after waiting for a long maceration period of 3 days

Effect clarification methods

In this study centrifugation and other physical separation of abomasal tissue were not significantly different ($P>0.05$), although a centrifugation speed of 3000 *g* for 15 min and gravitational separation of chemically activated tissue obtained higher MCA than

others. Filtering gave clearer extracts than other methods but used a lot of time to filter the extracts, therefore it was not recommended for SSCP.

Although $Na_2HPO_4.H_2O$ and Na_2HPO_4 were the same chemical, the former was more soluble. They were more efficient in separating the abomasal tissue from the enzyme than other chemicals. Furthermore, of the other chemicals used in this study NaOH is corrosive which requires more care in handling and Na_2CO_3 gives a lot of foam due to CO_2 production during clarification thus if they are to be used in case of emergency, their effects must be considered. Therefore the use of $Na_2HPO_4.H_2O$ and Na_2HPO_4 for clarification was recommended for SSCP.

Effect of storage temperatures

The significant difference ($P<0.001$) in activity of HP extracts stored at deep freezing temperature showed that extracts stored at these temperatures gave highest enzymatic activity as compared to those kept under ambient or refrigeration temperatures. Similar findings were reported by Kassell and Meitner (1970) who indicated that pepsinogen solutions be stored at -20^0 C because they are very sensitive to incidental activation by acid, denaturation and bacterial contamination which could have lowered the activity of extracts stored at room temperatures as seen in this study. However, they suggested that crude extracts must be stored in portions which can be used at once to avoid re-thawing and recontamination by microbes.

Effect of interaction of extraction conditions

Interaction of day x temperature and day x pH on MCA found in this study was not suprising and so was the interaction between NaCl concentration x pH and temperature x pH. This is because temperature, pH and ionic strength singly or in combination can affect MCA. Similar conclusion has been reported by Whitaker (1972) and Nelson (1975). Therefore if studying the effects of one of these factors the other parameters, must be taken into consideration.

5.1.2 Effect of different extraction solutions

In the present study, extracts had different colours, smell, taste and turbidity depending on the type of chemicals used in extraction. However, the salient observation was that extracts HP and STD were more turbid than others and this is probably due to different chemical composition of these extracts and chemical reactions with the abomasa. A similar reasoning was given by Peralta *et al.* (1986)

The pH and acidity of the extracts

In this study HP and VP results were similar to those reported by Peralta *et al.* (1986) who found the pH range of extracts from DTRI-IFS-6 to be 3.96 and 4.35 while the control had pH 5.85. However, the pH and citric acid content of LP was much lower because the lemon juice used as extraction solution was a weak acid of pH 2.4 and 0.22% citric acid content which reduced its shelf life while VP had more acetic acid (12.5%) which increased the preservative effect of the extract.

The MCA of the extracts

In the present study HP, LP and VP extracts of about 0.2, 0.5 and 0.3 ml respectively were found to coagulate one litre of cheese milk and made ready for cutting at 30^0 C in 40 min. This finding was different from that of Davide (1983) who used 4-5 ml of his extract per one litre of cheese milk. The differences could be due to different ratio of extraction solution to abomasa, the kind of extraction solution, pH and temperature of cheese milk which was different from that which was used in this study. Decrease in MCA of extract during storage was in agreement with findings of Kassell and Meitner (1970) who reported that they were susceptible to denaturation. Therefore before using the extracts for cheesemaking, a simple clotting test was recommended to check the extract MCA so as to apply the correct amount of extract to the cheese milk.

iii) Protein concentration of the extracts

Total protein content of the extracts of 1.2, 1.3 and 1.1 % for HP, LP and VP, respectively, were within the range of those reported by Davide *et al.* (1982) who found 0.83 and 1.29% protein in adult carabao and cattle, respectively. Peralta *et al.* (1986) suggested that the total protein values of the extracts could not be attributed solely to the enzyme extracted. Other nitrogenous material must have been extracted during the maceration and contributed to the crude protein content of the extract. This is in agreement with the present study because different extraction solutions were used, therefore varying amounts of nitrogenous materials in the extracts were expected.

Therefore the protein content of extracts was affected by the extraction solution used.

Microbial quality of the extracts

Microbial problems in the present study was only observed with the LP extract and this could mainly be due to a modestly low pH and acidity (pH 2.4 and 0.224% citric acid). It has been found by other workers that such conditions favour acid tolerant microbes especially yeasts and moulds. The other extracts were of good microbial quality although the pH was low and NaCl content was 10%. Similar findings have been reported by Peralta *et al.* (1986); Walstra *et al.* (1993) who found low counts of microbes in the extracts. The good microbial quality of HP and VP makes them suitable for cheese making.

Chymosin and pepsin isolates (fractions) by DEAE cellulose

The low content of chymosin ratio in the extracts as determined by DEAE cellulose were close to findings by Andren *et al.* (1981) who reported the ratio of 2:98 respectively in 12-24 months old heifers and traces for cows older than 24 months. Reasons for such differences were given by Hagyard and Davey (1974); Garnot *et al.* (1977) and Andren *et al.* (1981) to be strongly influenced by age, feeding regime, last feeding before slaughter, individual ability to produce enzyme and both daily and individual variations in the enzymatic activities of the abomasal juices.

However, extraction solutions used in their study were quite different from those used in this study, therefore some differences in MCA was expected as suggested by Rothe *et al*, (1976) who indicated that there is some lack of reliability in comparing activity ratios, as values of specific activity differ from one laboratory to the other due to different pH,

temperature and substrate in the milk clotting tests. Although the chymosin was low in these extracts, better levels were found in HP and VP implying that they were better rennet substitutes than LP

Effect of storage temperatures and type of containers

In this study the MCA of extracts kept at different temperatures and in different types of containers was fluctuating with storage time. Similar findings were reported by Herriot (1938); Lyatuu (1991) who indicated that as extracts are stored longer, their dissociable pepsin inhibitors liberated during activation of pepsinogen increase or decrease their proportion of the enzyme in the extract. Moreover, the changes in microbial activity in the extracts could lead to changes in MCA as suggested by James and Sielecki (1986) and Foltmann (1993). No literature information have shown the effect of glass and plastic containers on extract but could be due to the fact that plastic containers are permeable to oxygen and other gasses. These could have been absorbed into the extract, oxidized and denatured the extract. These can occur more at room temperatures like the HP in this study.

The natural volatility of vinegar and rapid loss of its water of hydration according to Dawson *et al.*1969) prompts one to suggest much loss of MCA when kept in plastic container and stored in freezer but less reactions when stored at room and refrigeration temperatures. Standard rennet, when stored in glass containers at freezer and refrigerator temperatures more than a year showed no quantitative change in MCA. But when stored at room temperatures in plastic containers it lost some activity. However, MCA did not

decrease as in HP when stored at room and freezer temperatures. This could be because the STD rennet was more purified than the extracts and NaCl content was 18% as compared to 10% in HP, LP and VP extracts, thus increased the preservative effect and its shelf life. Similar finding by Andren *et al.* (1981) indicated that 18% NaCl content in the extract prolonged its shelf life even at room temperatures. Therefore both glass and plastic containers were suitable for storing STD rennet extract.

Plastic containers could be used to store HP in the freezer and for refrigeration but not at room temperature while VP may be stored in plastic containers at room temperature and refrigeration but not in the freezer. Plastic containers seem to have some reaction with the VP extracts when stored at freezer temperatures.

Temperature and pH sensitivity of the extracts

Findings on sensitivity variations of extracts as compared with STD towards pH and temperature agrees with those of Davide *et al.* (1982); Andren and Reedtz (1990) who showed that to be due to the high content of pepsin. Pepsin was found to be more sensitive to pH changes than chymosin. Both HP and VP contained higher amounts of pepsin (96.5 and 97.4 % respectively), than STD but they varied in pH and temperature. Andren, (1985) found that the variations in sensitivities were due to differences in enzymatic chemical composition and concentration of the abomasal extracts. The stability of HP and VP at pH 5.0-6.5 and temperature of 28-38^0 C were appropriate conditions for normal renneting and cheese making so that the SSCP will not need to modify the conditions. Similar findings were reported by Davide *et al.* (1982). Therefore

extracts in this study had the right range of pH and temperature normally used in cheese making.

Comparison of formagraph characteristics of the extracts

The coagulation characteristics, where k_{20} curd firming rate was highest with STD which was not significantly different from VP ($P<0.05$) was in agreement with the findings of MacMahon and Brown (1985) and Andren and Reedtz (1990) who indicated that chymosin had faster curd firming rate than pepsin and other coagulants. But LP had shorter amplitude at 30 min. Similar results were found by Ortiz de Apodaca *et al.* (1994) who reported that vegetable coagulants gave lower value for a_{30} and observed that the coagulants tended to form a softer curd. However, this effect was not measured in this study. The longer clotting times of extracts than STD were in agreement with the findings of Andren and Reedtz (1990) who suggested that curd firmness in cheese is only a question of time since pepsin will give the same degree of curd firmness as chymosin after some minutes at pH 6.3-6.5.

Proteolytic activity of the extracts on αs1-casein (NPN)

In this study, the proteolytic activity of the extracts agreed with those of Preetha and Boopathy (1994); Ustunol-Ze and Zeckzer (1996) who reported that the ratio of clotting activity / proteolytic activity of chymosin was much higher than the corresponding ratio of pepsin. Moreover, Martin *et al.* (1981) estimated the clotting activity of chymosin to be twice that of bovine pepsin which was not investigated in this study, and all the

extracts used contained high amounts of pepsin. The protein breakdown by LP was highest, followed by HP and finally VP which was close to that of the control. Since there was no difference in incubation temperature and content of casein, it showed that LP was more proteolytic and VP was least proteolytic. But when HP and VP were compared on α_{s1}-casein HP was more proteolytic than VP. Therefore LP was less suitable for cheese making.

Proteolytic activity of extracts by agar diffusion diameter

Large inner transparent zone on agar diffusion diameter produced by LP was in agreement with findings of Lawrence and Sanderson (1969) who suggested that enzyme producing larger inner transparent zone relative to white outer zone would be unlikely to make suitable milk clotting agent in cheese manufacture as it would further degrade the clot. He therefore suggested the selection of proteinases which gave a larger precipitate but comparatively small inner transparent zones as was found in this study for HP, VP and STD. Therefore the VP and HP were more suitable for cheese making than LP.

Separation of peptides released from casein by HP and VP extracts

In this study the Reversed Phase -FPLC chromatograms showed peaks of peptides and proteins separated with acetonitrile gradient also for the un-incubated sample containing presumably peptides and proteins present in the extract. The number of peptides increased markedly in the incubated samples and new peptides eluted after shorter separation time appeared in HP and VP extracts. Some differences in the peptide profiles

may indicate differences in both specificity and activity between VP and HP extracts, therefore, HP was more proteolytic than VP. The arrows in Figure 12 A, B, C marks the elution time of α_{s1}-casein fragment 1-23 which was a major peptide released by chymosin or pepsin from α_{s1}-casein as was reported by Fox and Stepaniak (1993); Visser (1993); Singh *et al.* (1995) Fox *et al.* (1995). The peptides released by the extracts were not determined in this study.

5.1.3 Effect of enzyme concentration

In normal conditions MCA should increase with decrease in moisture content which was the case for HP when concentrated to constant weight. However, STD lost some MCA and VP lost all MCA. Similar findings were reported by Soral-Smietana and Muzinska (1994) who reported that drying the mixture of a high water content ccould lead to significant deformation of their microstructure and therefore lead to false interpretation of the results obtained which could be true for VP in the present study. Moreover, Dawson *et al.* (1969) showed that vinegar is a dilute acetic, carboxylic, ethanolic acid which is volatile, has a high pKa of 4.64 and looses water of crystallisation. Therefore it is possible that when it was concentrated, the enzyme pepsin changed its structure when loosing water of crystallisation leading to enzyme denaturation and loss of activity. Therefore, it was recommended that VP always be kept in liquid form in order to maintain its MCA.

5.2 Experiment 2: purification of bovine pepsin

5.2.1 Purification of crude pepsin on DEAE cellulose

The resolved peaks of chymosin and pepsin as in step 1 of purification of STD and HP were also reported by Garnot *et al.* (1972) and Rothe *et al.* (1976) who observed well separated fractions of pepsin and chymosin during purification of commercial rennet n DEAE cellulose. They demonstrated by measurement of the proteolytic activity /MCA ratio that chymosin was eluted in the first peak and pepsin in the second peak. Garnot *et al.* (1972) recommended for best separation of the two enzymes, anion exchange chromatography on DEAE cellulose. The height of the peaks of HP extract indicated that chymosin was present in a small amount while the pepsin content was much greater than that of chymosin. The peaks of MCA at pH 6.0, 6.4 and proteolytic activity measured by agar diffusion method (AD) were well superimposed but AD seem to be very sensitive thus the activity in the fractions were represented by long coagulating times. This result agrees with those of Cheeseman (1963). Lawrence and Sanderson, (1969) also reported that AD was considerably more sensitive than MCA. MCA of milk at pH 6.0 was higher than at pH 6.4. This indicated the importance of standardisation of milk acidity when working with pepsin extracts. Foltmann (1987) reported that pepsin is more sensitive to pH changes than chymosin and its activity may also be more dependent on the experimental conditions like ionic strength of solutions, temperature, duration of the experiment and concentration of enzymes.

The first step of pepsin purification resulted in an increase of 26 MCA/mg protein specific activity (Table 12).

5.2.2 Purification on Gel filtration

Elution of pepsin and chymosin from the Gel filtration column were in different fractions indicating some difference in molecular masses. Some impurities of higher and lower molecular mass than of pepsin and chymosin were eluted indicating the need for further purification. Castle and Wheelock (1971) purified chymosin from rennet using the same type of column. They noted a protein peak eluted in the void volume which suprisingly contained a small proportion of polymerized chymosin and pepsin which did not have clotting activity.

When the sample was applied on SDS-PAGE, a single band of pepsin was eluted. Amourache and Vijayalakshmi (1984) reported that the above methods were much used in estimating the molecular mass of enzymes when compared with the protein standards.

5.2.3 Purification by Mono Q anion exchanger on FPLC

Pepsin fraction was eluted from the Mono Q column at the end of the gradient when the NaCl concentration was 0.5 M. A small peak without MCA or AD was eluted before the main protein peak. The main peak of protein was quite symmetric and the MCA was well superimposed under the protein peak. According to Whitaker (1972) such chromatographic behaviour is a criterion of a homogeneous or pure preparation. Since a small peak without MCA or AD was observed before the main peak showing that there was still some impurities, the fraction had to be further purified.

5.2.4 Purification by Phenyl Sepharose on FPLC

Occurrence of the three partially separated peaks with MCA after Phenyl Sepharose was similar to the findings by Rajogopalan *et al.* (1996) who separated well commercial rennet with phosphate buffer at pH 5.7 by chromatography on hydroxylapatite, obtaining two peaks of active material. Perhaps lower than 1.7 M ammonium sulphate concentration could result in a better separation of pepsins by hydrophobic interaction chromatography on Phenyl Sepharose. Meitner and Kassell (1971) reported that all the 3 separated pepsin peaks on hydroxyapatite at pH 7.4 had N-terminal valine, C-terminal alanine and were free from carbohydrate. The only difference they detected among them was the organic phosphate content value ranging from 0.3 to 3 mol/mol. They showed that the phosphate content accounted for the 3 peaks of chromatographic separation. In the present study results do not confirm valine as N-terminal amino acid, but they indicate varying mass of pepsin with different degree of phosphorylzation

5.2.5 Rechromatographed pepsin on Mono Q

The rechromatographed (A) single sharp peak obtained was also reported by Rajagopalan *et al.* (1996) who rechromatographed the two well separated peaks on hydroxylapatite after equilibrating against phosphate buffer of 0.15 M of pH 5.7 and observed the first single sharp peak which emerged at 0.2 M. Moreover Nielsen and Foltmann (1993) when purifying porcine pepsin on ion exchange chromatography observed a single separate band of pepsin on SDS-PAGE, which was also found in this study and they concluded that the contents of the peaks were partly unfolded pepsin in

which some activity has survived the chromatography at pH 7. Such pepsin species may be cleaved from complexes which have dissociated after application to the column. Therefore the pepsin preparation was homogeneous at step III.

5.2.6 Characterisation of purified enzyme

Electrophoresis.

Appearance of a single and well separated band of protein in SDS PAGE of peak activity fractions from Mono Q and Mono Q rechromatography indicated that the high purity was attained at Mono Q step. This result agrees with those of Garnot *et al.* (1972); Fox (1987) and Foltmann (1987).

Molecular mass of pepsin and chymosin.

The molecular mass of pepsin and chymosin in gel filtration and SDS PAGE were 34,000 and 34,500 Da, respectively. These results are lower to those of Deyl (1979) and Szeisi (1992) who reported the molecular mass of calf chymosin and bovine pepsin to be 35,600 and 35,000 Da respectively. Foltmann (1970); Rye (1972) and Szecsi (1992) got nearly the same results while Abdel Malak *et al.* (1996) got MM 36,000 Da when he purified buffalo chymosin by Gel filtration.

Amino acid sequence N-terminal of pepsin

The evaluation of amino acid sequences by Swiss- Proc. Data Base showed that 7 amino acids of the N-terminus of our purified pepsin matched the sequence 48 - 55 of bovine

pepsinogen confirming the identity of the enzyme, the N-terminus indicated that the peptide of 48 amino acids had been cleaved off and the amino acid sequence identified was Ileucine-glycine-phenylalanine-leucine-glycine-aspartic acid-tyrosine-leucine (Ile-gly-phen-leu-gly-asp-tyr-leu).

Lysine was not identified in this study which is in accordance with Nevaldine and Kassel (1971); Foltmann (1987) and Abdel Malak *et al.* (1996) who reported that lysine should be absent in purified pepsin to prove the purity of the sample.

5.3 Experiment 3: Assessment of cheesemaking qualities of HP and VP extracts

5.3.1 Renneting times of cheese milk, TN and BF in whey for SUA Alpine and SUA Pasta

Renneting times

The renneting times of milk with CR/pepsin combinations for SUA Alpine and SUA Pastafilata cheeses were within the acceptable range of 40 - 60 min commonly used in cheesemaking as reported by Lenoir and Schneid (1987). RT in SUA Alpine cheeses increased with increasing relative amount of pepsin and 100HP, 100VP and 75VP had the longest RT. RT in SUA Pastafilata cheese were higher in combinations with high pepsin levels although they were not directly related to level of enzyme but RT were longer than for the controls. Similar findings by Andren and Reedtz (1990) indicated that chymosin had shorter gelling times than pepsin. Therefore when pepsin is used for cheesemaking without rennet recommended RT will be longer than that of rennet.

Total nitrogen (TN) in whey

The TN loss in whey of cheeses made with different levels of pepsin, was within that reported by Bank *et al.* (1993) who found it to be more than 20%. The lower TN loss in CR cheese whey than TN in cheese whey of other pepsin levels was in agreement with the findings of Hill (1995) who reported that most TN loss occurred during and after cutting of the coagulum. The TN loss in SUA Alpine whey was directly related to the level of pepsin showing that pepsin was more proteolytic and especially the VP combinations which had the highest TN losses in whey. Moreover cheeses with high levels of pepsin had longer RT which meant that curd firmness decreased as the level of pepsin increased. Therefore, during cutting and after cutting and stirring of curds, high losses of TN in whey could have been increased with high levels of pepsin in these cheeses than others. The statement that high TN loss occurred more during and after cutting was reported by Holsinger *et al.* (1995). Higher losses of TN in SUA Pastafilata whey may have been because the milk was not pasteurised, and similar findings by Hill (1989, 1995) indicated that during milk pasteurisation, whey proteins denature and aggregates and enter complexes with κ-casein thus increasing the transfer of TN into cheese.

Butterfat (BF) in whey

Butterfat loses in whey for SUA Alpine and Pastafilata cheese made with CR/pepsin combinations especially SUA Alpine 100VP, SUA Pasta 100VP and SUA Pasta 100HP were quite high. Similar findings were reported by Barbano and Rasmussen (1992) that

the use of bovine pepsin was conducive to more loss of BF in whey than when other coagulants were applied. However, temperature used in cheese moulding was also important whereas the temperatures used in cheesemaking varied very little thus levels of pepsin was the most important for more loss of BF. The BF losses in Alpine cheese ranged from 6% for 50HP, 75VP cheeses and 18% for 100VP cheese (calculated from Table 15) These results were similar to those reported by Phelan *et al.* (1981) and Van Boekel (1993) who showed that fat recovery in many types of cheese ranged from 85% to 93% or BF loss from 7 to 15% in whey which also depended on the mechanical treatment during the cheesemaking process. Hill (1995) suggested that BF loss in whey could be reduced by allowing sufficient time for cut edges of curd particles to heal before further processes continue. Therefore extended time for "healing" could reduce BF loss in cheese with high pepsin levels. High losses BF in Pasta was due to high temperatures used in moulding the cheese.

5.3.2 Chemical composition of cheese

pH

The slow trend of pH increased during ripening of SUA Alpine, village Alpine and Tilsiter cheese was also reported by McSweeney and Fox (1993) and Walstra *et al.* (1993) who found that during ripening of Dutch cheese, the pH increased slowly primarily due to alkaline nitrogenous and buffering compounds of Ca-paracasein and Ca-phosphate complexes being solubilized (Holsinger 1995). Also the early stages of cheese ripening lactose was slowly being converted into lactic acid which then affect the

pH of cheese. By 42 days, the pH ranged 5.2 - 5.58; 5.9 - 6.36 and 5.0 - 5.61 respectively. The pH values for SUA Alpine and Tilsiter agrees with that of Walstra *et al.* (1993) with pH 5.0 - 5.6. Findings by Walstra *et al.* (1993) that high pH lead to high moisture and low pH lead to increase in syneresis in cheese agree with the finding in this study where SUA Alpine 100HP cheese of highest pH 5.58 had the highest moisture content of 40.1% while 25 VP cheese had lowest pH of 5.2 and its moisture content was 31.7%; Tilsiter 75VP cheese of pH 5.4 had 30% moisture content while 50HP and 50VP cheese of pH 5.0 had 25 and 26 % moisture, respectively. High pH values observed in some cheese, especially those with high levels of pepsin, may have been contributed by longer coagulation time of milk. Similar findings were reported by Medina *et al.* (1992). High SPC, yeast and mould counts in village Alpine cheese especially day 2 may have contributed to high pH values due to the microbial metabolites; similar findings were reported by Walstra *et al.* (1993).

For SUA and village Pasta cheeses, at 4 days of maturity the pH values were within the standard and their pH ranges were very narrow indicating similarities in metabolic production. The reason for little acid production was reported by Steffen *et al.* (1993); Kindstedt (1993, 1996) found that the subjection to kneading and plasticising in this type of cheese in hot water at 60-70 ^{0} C killed most of the starter bacteria which produce lactic acid. Moreover they reported that the use of hot water during the process of moulding cheese was found to dilute lactose in the curd hence less lactic acid production (Desmazeaud, 1987). Also Kindstedt *et al.* (1993) pointed out that when extended time

of cooking and kneading was allowed, the curd lost Ca^{++} into whey (33 - 42%) which alternately lowered the pH of cheese. Determination of lactose and Ca^{++} left in cheese was not done in this study, but variations in moulding time of different cheeses in hot water was expected because it was manually done. Although pH varied in cheese this was not directly influenced by level of pepsin.

Acidity

Acid production trend in SUA Alpine, village Alpine and Tilsiter cheese was general and fairly rapid in 21 days of ripening and then rose substantially among cheeses. Similar findings were reported by Walstra *et al.* (1993) that acidity should proceed fairly quickly in the early stages of ripening due to lactose conversion to lactic acid and more slowly later on. Literature information does not clearly show what the standard acidity in cheese should be and also Fox *et al.* (1995) found that very few quantitative data have been published on this subject. Therefore, some variables in cheese composition cannot be explained satisfactorily especially the degree of acidification in cheese which showed unexplained fluctuations. Desmazeaud, (1987) suggested that the percentage acid should be more than 1.3 % while Walstra *et al.* (1993) suggested it to be around 3 %.

Comparison of cheese acidity values with their cheese controls, showed that SUA Alpine, village Alpine, SUA Pasta and village Pasta and some Tilsiter cheese had acidities close to their controls and the range was small showing good acid production in most cheeses which Holsinger *et al.* (1995) found to be the key for production of good quality cheese and preservation of cheese (Walstra *et al.*1993). Tilsiter cheese 50HP,

100VP, and 25VP had higher acid content than the control. However, these cheeses together with the control had higher microbial counts which were in agreement with findings by Walstra *et al.* (1993) that all cheese types first undergo lactic acid homo-fermentation with over 90% of the lactose being converted to lactic acid. Also, lactic acid produced by starter bacteria may be metabolised by organisms of the surface flora on soft cheese thereby increasing the pH (Steffen *et al.* 1993; McSweeney and Fox, 1993).

In village Pasta, acid production did not proceed far because much lactose could have been removed from the cheese during moulding of the cheese and fermentation. Acidification may have been stopped due to high temperatures used which might have destroyed many starter bacteria which produce lactic acid. The acidity of the control, 100HP village Pasta and 100 HP SUA Pasta did not increase and their NaCl contents were highest. Similar results have been found by Walstra *et al.* (1993) who reported that cheese brined soon after manufacture, early brining causes lactose to be left in the outer rind portion and lactic acid production was restrained by NaCl cooling which agree with the present study whereby cheeses were brined soon after manufacture and cooled.

Moisture

The trend of moisture in cheese decreasing with ripening time agrees with reports by Medina *et al.* (1992); Fox *et al.* (1993) and Walstra *et al.*(1993). The moisture content of mature cheeses SUA Alpine, village Alpine, Tilsiter, were lower than the standards reported by Chapman and Sharpe, (1981) and Walstra *et al.* (1993) to be 45-50% for

Dutch type cheese. Village Pasta cheeses had moisture content lower than that reported by Walstra *et al.* (1993) to be 45-52% for Swiss type cheese while SUA Pasta moisture contents was within acceptable range except cheese 25VP. Report by Holsinger *et al.* (1995) showed that the amount of moisture retained in cheese is influenced by the extent and combination of steps used in cheesemaking. All the cheese manufacturing steps were done manually in the present study, that is renneting, cutting, cooking, stirring, and NaCling and the intensity of treatments may therefore have differed somewhat from cheese to cheese leading to differences in moisture contents. Cutting the coagulam manually could lead to much variation of curd particle sizes resulting in differences in moisture content as reported by Hill (1995) , stirring of the curd was not uniform some curds may have been stirred more frequently and intensively than others which could again lead to some curds being more dry than others. Cooking temperatures for the present cheeses were normally 38-45^0 C and may have varied between batches, and high cooking temperature effects increased removal of moisture from the curd while low temperatures leaves more moisture which agrees with results of Hill (1995) and Kindstedt (1996). The present study agrees with all these conditions concerning variations in cutting, stirring and cooking. Pressing of SUA Alpine, village Alpine and Tilsiter cheeses was nearly equal with a pressure of 10kg /1 kg cheese, therefore this step should not have much effect on the moisture. Walstra *et al.* (1993) who reported on the standard moisture content in cheese did not indicate the pressure used in their cheesemaking which could lead to some inconsistency in comparing cheeses.

High moisture content in some cheese with high levels of pepsin and low moisture in some cheese with low pepsin levels for example SUA Alpine 100HP, village Alpine 100HP, SUA Pasta 100HP and 100VP had high moisture while SUA Alpine 25VP, village Alpine 25HP and 25VP and SUA Pasta 25 VP had low moisture indicating that level of pepsin had some influence on moisture content of cheese. These results disagree with those of Medina *et al.* (1992) which could be because they compared the recombinant chymosin with other coagulants but not levels of coagulant in Spanish cheese. During cheese ripening, humidity and temperature in cheese ripening room may influence the moisture loss. Therefore, humidity should be about 80% and tempature about 19 ^{0}C so as to avoid loss of moisture from cheese. Also covering cheeses with different materials could help in reducing the intensity of moisture loss from cheese (Stehle, 1987).

The method of drying the samples was the same but Bradley (1994) reported that moisture is not released uniformly over drying periods but its release is a function of whether water is bound or free and its location in the cheese matrix which could have lead to moisture variations in this study.

Butterfat (BF)

The general trend for BF in most of the cheeses decreased with maturity which agree with the findings by Chapman and Sharpe (1981) that during ripening fat was hydrolysed with the release of volatile short chain fatty acids by the action of starter

bacteria, residual milk lipase and heat stable lipase of psychrotrophic organisms thus decreasing the amount of BF.

Butterfat (in DM) in SUA Alpine, village Alpine and Tilsiter (Dutch type) with the ranges 43.8- 60.3, 43.4-57.8 and 43.1-52.7 %, respectively, were higher than the standard reported by Walstra *et al.* (1993) with 40-50%. In SUA Alpine, village Alpine and Tilsiter BF was on the higher side in control cheeses while cheeses with high levels of pepsin had BF on the lower side showing that the level of pepsin had some influence on BF content. The influence of pepsin level was even more pronounced in Tilsiter and SUA Pasta cheese where the higher the pepsin level, the lower was the BF.

In SUA Pasta and village past the BF ranged from 32.8 to 48.1% and 35.4 to 47.5%. The values were within those reported by Kindstedt (1993) to be 30 - 45%. In spite of the fact that the values were within or very close to the standard range, cheese 100HP, 100VP and 100VP in SUA Pasta and village Pasta respectively were much lower in BF than the others. Similar findings of Barbano and Rasmussen (1992) indicated that bovine pepsin caused more loss of BF in whey than did chymosin, however, the extent of loss also depended on the temperature used in cheese moulding. In the present study the BF in cheese milk was 3.0 to 3.3% but the values observed in the cheeses varied. Similar observations were reported by Walstra *et al.* (1993) who pointed out that under practical conditions of cheesemaking, establishment of correct fat content in milk caused specific problems because cheese was always inhomogenous. Bored samples could have given considerable bias from the loaf and the fat content of different loaves from one batch was not identical, the standard deviation was 0.5% DM. Therefore to some extent, the

level of pepsin in cheese was directly related to BF content of cheese especially in Tilsiter and SUA Pasta cheese.

NaCl

In most cheeses NaCl was low at the beginning and increased with ripening time because there was a process of diffusion of NaCl across the curd. This agreed with the findings by Steffen *et al.* (1993) who showed that both concentration and uniform distribution of NaCl took place throughout the cheese and were important for cheese quality.

The ranges of NaCl content in SUA Alpine, village Alpine, Tilsiter, SUA Pasta and village Pasta cheeses were 1.3 - 7.1, 1.6 - 4.3, 0.7 - 4.03, 1.5 - 5.9 and 1.2 - 5.5 %, respectively. Walstra *et al.* (1993) reported a range of 2 - 7% for Dutch type cheese and Kindstedt (1995) reported a range of 0.8 - 2.7% for Swiss type cheese. The SUA Alpine cheese except 50HP, village Alpine except 100CR and most Tilsiter, SUA Pasta and village Pasta cheese had high NaCl values and relatively low moisture contents indicating that NaCl had an influence on moisture content of cheese. Similar findings by Holsinger *et al.* (1995) showed that NaCl does not control the amount of moisture in cheese but regulates it.

Also Holsinger *et al.* (1995) reported that incorrect salting can lead to adverse effects on cheese quality because the greatest roles of NaCl in ripening is to control water activity, microbial growth, enzyme activity, physical changes in cheese protein that influence texture, and solubility through possible changes in protein conformation. Thus, too high

NaCl concentration will retard ripening and protein decomposition (Fox, 1987), and low NaCl will cause brittle body, cracking of rind, abnormal ripening and increased shrinkage in curing (Ling, 1963). Incorrect salting of cheese in this study did not occur because all cheeses were brined in 15 - 20% NaCl. Therefore these results showed that cheeses differ in the way of absorbing NaCl: 50HP SUA Alpine absorbed much less NaCl than the other cheeses; 100CR village Alpine and Tilsiter; 100VP, 25VP, 100HP in SUA Pastafilata; 100CR, 25HP, 25VP village Pasta, all these different cheese contained less NaCl than others which agrees with suggestions by Fox *et al.* (1995) that cheese differ in their microscopic structure and hence their rate of NaCl absorption and diffusion. The NaCl content of cheese was not directly related to the level of enzyme.

Total nitrogen

Normally in a given cheese there is an amount of nitrogen which does not change much. However, in the present experiments notable changes were observed. Degradation of proteins or even peptides does not change TN. Change in TN occur only if the amino acids are further degraded and disappear as volatile ammonia or are used by microorganisms present in cheeses. The high microbial counts especially at 28 and 56 days due to overipening of cheeses could have contributed to some proteolysis thus leading to lower TN. Similar observations were reported by Choisy *et al.* (1987) that heavy microbial activity and their proteolytic enzymes in cheese can lead to breakdown of TN. However, the increase of TN in most cheeses with ripening time was reported by

Hardy, (1987) who showed that in most cases it was influenced by moisture changes during cheese ripening.

But in this study TN was converted into dry matter content and amounts of TN did not vary much with cheese maturity.

Water soluble nitrogen (WSN) and ripening index (RI)

Generally WSN increased with ripening time in SUA Alpine, village Alpine and Tilsiter. Similar findings by Medina *et al.* (1992); Barbano *et al.* (1993); Walstra *et al.* (1993); Fox *et al.* (1995) and Kindstedtz *et al.* (1996) showed that the results were basically due to the residue coagulant which was responsible for initial hydrolysis in cheeses characterised by rapid degradation of α-s-casein in early maturity of cheese, whereby 80% of α-s-casein was hydrolysed within one month and then β-casein was degraded more slowly for a longer time. In the present study, levels of residual coagulants were not measured, however, Fox *et al.* (1995) reported that 6% of chymosin added to cheese milk was retained in cheddar cheese. The WSN values varied much with levels of pepsin. No apparent direct relationship occurred between WSN and enzyme combinations in this study. Similar conclusions were drawn by McSweeney *et al.* (1993), who pointed out that proteolysis measured by WSN may not be sufficiently discriminating as an index of quality. Literature have not shown the standard values for WSN although Walstra *et al.* (1993) indicated values for WSN in Gouda and Mozzarella cheese which were extracted at pH 4.5 and with 12% TCA to be 20% and 13.9 % and 7.3% and 2.4% respectively while McSweeney and Fox, (1995) reported 25% in mature

Cheddar cheese. Kindstedtz *et al.* (1996) reported an average of 4.07% for the first day and 9.66% after 14 days of ripening low moisture Mozzarella cheese while Fox (1993) found 3.65 - 13.12% WSN. The values of WSN as percentage of TN of the cheeses in this study were within the reported values. The WSN values within the type of cheese varied very much. These variations of WSN is in agreement with the report by Walstra *et al.* (1993) and Fox *et al.* (1990, 1995). Moreover, similar findings by Visser (1977); Walstra *et al.* (1993) and Fox *et al.* (1995) indicated that proteolysis was the most complex and perhaps the most important biochemical event during the ripening of most varieties of cheese. It varied from limited to very extensive like in village Pasta cheese 100HP to 100VP and Tilsiter 25VP to 25 HP. Farkey (1995) and McSweeney and Fox (1995) showed that the rate and extent of hydrolysis or proteolysis was determined by the type of cheese, amount and activities of the proteolytic enzymes present in cheese.

Walstra *et al.* (1993) and Fox *et al.* (1995) reported that NaCl content, pH and moisture can affect cheeses ripening or proteolysis. NaCl content in most cheese in the present study were within standards levels, except in SUA Alpine 50VP, village Alpine 100CR, Tilsiter 100CR, 100HP, 75VP, SUA Pasta 50HP, 75HP and village Pasta 100VP, 75VP which had lower NaCl content but did not have high WSN compared to others within the type of cheese except in SUA Pasta 50HP where NaCl was lowest but WSN was highest showing that there was a relationship with NaCl content in this cheese.

Most cheeses had pH within or close to the standard except in village Alpine. SUA Pasta cheeses had higher pH and some of them had high WSN which was not directly related to the level of pepsin i.e. cheese village Alpine 25HP had pH 6.36 but WSN was

18 % while 75VP had pH 5.85 but WSN was 21 %, Tilsiter 75VP had pH 5.61 with WSN 24% and 25HP with pH 5.02 had 5.8% WSN. So pH could have contributed to high WSN production in these cheeses but not in all cheeses because some cheeses which had high pH had low or equal WSN values i.e. village Alpine 25HP with pH of 6.36 had 18 % while 50VP with pH of 6.03 had 17.7% WSN. Moisture content of most cheeses was below standard values, therefore it would not be expected to contribute much to proteolysis (Visser, 1977; Walstra *et al.* 1993 and Fox *et al.*1995).

The higher values of WSN in some Tilsiter cheeses could mean that there was more hydrolysis of casein. This could have partly been due to the presence of indigenous proteinase an acid zymogen procathepsin D found in raw milk as reported by Kaminogawa *et al.* (1980); Fox, (1995). The proteinase could have contributed to more proteolysis in these cheese because half of the cheese milk used was raw.

WSN values in SUA Pasta and village Pasta cheeses were on the lower side than those of Dutch type cheeses. This was in agreement with findings of Fox *et al.* (1995) ho indicated that the coagulants were extensively or completely denatured by high cooking temperatures so the contribution of plasmid to initial hydrolysis of casein was more pronounced in these types of cheese than the coagulant. However, in this study plasmids in cheeses were not determined.

It can be concluded that WSN as a percent of TN varied with variety of cheeses and no direct relationship with level of pepsin was found in the present study.

5.3.3 Microbial quality

Standard plate count (SPC)

At the beginning of ripening SPC in cheeses was high in most cheese. Mertley and Crow (1993) and (McSweeney *et al.* (1993) reported this to be due to deliberately added starter, secondary starter, thermoduric organisms from milk and adventitious contamination some of which survive pasteurisation and grow rapidly in cheese a few hours after manufacture (3 hours). At this time lactic acid is low, pH is high, moisture content is high and NaCl content is low. All these conditions favour the growth of even some undesirable organism leading to high counts (Walstra *et al.* 1993, Kindstedt 1995, Fox *et al.* 1995). As the cheese started ripening, the bacterial counts decreased. Similar results by McSweeney *et al.* (1993) and Martely and Crow (1993) showed that the decrease was due to the decline in lactose as growth substrate. Starter numbers declined due to the combination of the absence of lactose, low pH and high sodium chloride concentration in the cheese. The decrease in bacterial counts in cheese due to decrease in pH of cheese agree with results by Walstra *et al.* (1993); Steele (1993); Zottola and Smith (1993) and Chapman and Sharpe (1981) who showed that low pH of 5.0 - 5.2 helped to suppress the growth of pathogens and spoilage bacteria in cheese. The SPC of SUA Alpine, village Alpine and Tilsiter cheese, increased later when the cheeses were left to overripe to 56 days. However, as ripening continued the NaCl content of cheese increased and moisture decreased. The two factors were reported by Zottola and Smith (1993) to be the most important and effective in controlling the growth of undesirable microoganisms in cheese. Fox (1993) reported that a NaCl content of 4 - 5% reduced the

microbial protease's to 50 - 80% compared to NaCl free cheese. This agree with the present study in which most cheeses had low NaCl content had high counts i.e. SUA Alpine 50VP, control village Alpine, 50HP SUA Pasta, control Tilsiter and 75VP Tilsiter. The total counts of cheese at maturity were within those reported by Steffen *et al.* (1993) who found 3.5 x 10^5 cfu/g and Fox (1993) who reported that great numbers of microbial species are found in cheese, the total number generally exceeding 10^7 cfu/g. The variations in counts of different cheeses were due to differences in pH, moisture content, redox potential and temperature of the cheese. In this study high hygienic conditions were closely observed to avoid contamination of cheese. However, Zottola and Smith (1993) stated that inspite of the hygienic standards, contamination of milk and curd with undesirable organisms cannot be fully prevented. Counts in Tilsiter varied much and were higher than in other cheeses and as indicated above, cheeses were made from half raw milk. Again milk was collected from different farmers with varying hygienic standards. Similar findings of Martely and Crow, (1993) indicated that 50% of the micro-oganism in milk were NSLAB strains of leuconostocs, pediococci and mesophilic lactobacilli which are most heat resistant. The counts especially the lactobacilli are initially low but rapidly increase depending on the cheese variety. Depending on the ripening times they utilise the released intercellular enzymes and cellular components such as sugars and nucleic acid from the lysed starters and adjuncts for growth. The increase of SPC after maturity shows that it is important to use cheese as soon as possible or market them just before maturity

In SUA Pasta and village Pasta, the counts in cheese were relatively low at the beginning of ripening because of the high moulding temperatures (*Kindstedt et al. 1993*). Low pH, low NaCl and high moisture allow starter cultures, adjuncts, undesirable organisms that survived pasteurisation or which recontaminate the curd to start growing in the young cheese. Again counts had increased by day 28 which Zottola and Smith (1993) showed to be due to the de

velopment of soft pastry on high moist surfaces during ripening caused by diffusion of water from the low NaCl concentration centre of cheese to the surface which had high concentration of NaCl thus creating favourable conditions for growth of microbes.

Coliforms

The coliform counts were high in the beginning and at the end of cheese ripening. Walstra *et al.* (1993) showed that coliforms require lactose for growth and multiply rapidly in a few hours when the pH and temperature is favourable and then later decrease with ripening time of cheese. Most of the cheeses at maturity had few coliforms especially SUA Pasta cheeses. However, coliform counts in some cheeses increased after 42 days showing that there was some contamination. Similar findings by Walstra *et al.* (1993) indicated that coliforms were killed very fast at pasteurizing temperatures but they easily recontaminated during cheesemaking. The fact that cheese recontamination may occur was confirmed in this study where movement of cheeses was made necessary in order to acquire enough space on the shelves for all cheeses produced within a short time. Low counts of coliform at maturity was also reported by Zottola

and Smith (1993) who found that the decrease was due to production of lactic acid on which even cheese manufacturers rely to compete out the coliforms.

Yeast and mould

The trend for yeasts and moulds in cheese was similar to that of SPC. Cheese controls had the lowest counts except for Tilsiter which had highest. Walstra *et al.* (1993) reported that the main source of lactobacillus, yeasts and moulds in cheese was the brine deposit, where NaCl-tolerant yeasts can survive in its pH, a decreasing NaCl concentration and higher temperature in the cheese than that of the brine, inadequate drying of the cheese rind after brining and insufficiently cleaned shelves in the ripening room. The last two sources might have been the main sources of recontamination in this study. During cheesemaking, there were too many cheeses and few shelves for cheese storage. This forced a lot of movement of cheese especially for village made cheeses. The brine contained 15 % NaCl which according to Fox *et al.* (1995) was quite sufficient, but was occasionally changed which might have led to the contamination by NaCl- tolerant yeasts on the cheese surfaces. At overripened stage, moulds and yeasts can still grow in low moisture cheese. Moreover, Chapman and Sharpe (1981) indicated that the lower the pH of cheese the better are the conditions for yeast and mould to grow and compete with other microorganisms

SUA and village Pasta were cooled in water before salting and Zottola and Smith (1993) showed that cooling water is one of the main sources of contamination of cheese surfaces. Again in most places were cheeses where made, there were cows near-by and

Zottola and Smith (1993) indicated that yeasts and moulds can come from manure and bedding materials to milk and cheese. The number of microbial counts in cheese were not related to the level of pepsin.

5.3.4 Sensory evaluation of cheese

Taste

The non significant differences ($P>0.05$) in taste of different cheeses was in agreement with the findings of de Berg and de Koning (1990) that sometimes minor variations in composition occur between individual cheese even in the same batch, which may cause slight quality differences and in a few cases the results are significantly different in total preference score between the two cheeses being assessed as a pair. Such differences were mainly due to slight differences in intensity of the acid taste of cheese which has no relation to the rennet used. The acid content of SUA Alpine cheese which were significantly different ($P<0.001$) in taste could have lead to low scores in those cheeses especially 100CR and 100VP, 25VP which produced more acid. However, this was not true to all cheeses because 25HP also produced high acid but the taste score was high. Similar results were reported by Walstra *et al.* (1993) that flavour development due to breakdown of lactose to lactic acid caused refreshing acid taste but excess was shown to render the cheese sour. Diacetyl was reported to affect the aroma and taste. Moreover, other numerous secondary products formed during fermentation such as, ketones, alcohols, esters, organic acids and CO_2 affected the flavour of the cheese (Walstra *et al.* 1993; Steele, 1995).

SUA Alpine cheese with low taste scores such as 50VP and 25VP had low NaCl content, but 75HP with high NaCl content had high taste score showing that NaCl contributes to taste in cheese. This was in agreement with findings of Hardy (1987) who reported that NaCl imparted characteristic taste and enhanced or masked cheese taste flavour of certain substances that appear as the cheese mature.

SUA Alpine 25VP with low BF had a low score in taste while 75VP had high BF and high score showing that BF contributed to taste of cheese. Kindstedt *et al.* (1993) reported that low fat cheese had bland taste. NSLAB which have been reported to have significant influence on cheese flavour development (Steele, 1995) could have caused cheese 50HP and 50VP to have low taste scores because these cheeses had higher microbial counts, however, NSLAB were not determined in this study. Present results especially with HP cheese combinations showed a direct relationship between taste score and level of pepsin which agrees with findings of Steele (1995) that the level and specificity of the coagulant influence the flavour of cheese although Andersson and Andren (1990) showed that using different ratios of chymosin/ pepsin for cheesemaking was not a problem.

Smell

The smell of all cheeses were not significantly different ($P>0.05$). This could mean that there was a correct balance of flavour compounds in all cheeses. Steele (1995) and Fox *et al.* (1995) reported that for development of good flavour, a correct balance termed as

'component of balance theory' must exist between various flavour substances. Walstra *et al.* (1993); Fox *et al.* (1995) reported that if there is excessive lypolysis, free fatty acids soon produce rancid flavour and excessive proteolysis due to breakdown of paracasein into amino acids which are further degraded into ammonia, hydrogen sulphide produce objectionable strong smell. Lypolysis apparently was not a problem in this study. In addition Zottola and Smith (1993) also reported that coliforms or any other lactose-fermenting organisms if present in excess in cheese, may produce gaseous cheese and cause undesirable flavour. Fortunately there were only low counts of coliforms in most cheeses especially at maturity. It can be concluded that all cheeses were properly ripened without apparent flavour defects.

Appearance and texture

Findings of Steffen *et al.* (1993) indicated that extensive proteolysis cause various defects in cheese. However, too little proteolysis lead to flat taste, poor body consistency, uneven ripening which might be true for SUA Pasta which was tested after 14 days instead of 21 days although the maturity time ranges from 14 - 21 days. Also Zottola and Smith (1993) reported that undesirable organisms can lead to alteration of texture, flavour and body appearance in cheese and this could be true for SUA Pasta 25VP which had high SPC and yeast/mould counts and scored lower in appearance. So the two reasons could have affected the cheese but were not directily related to the number of microbial counts in cheese. Therefore SUA Pasta could have been evaluated at 21 days at which it might have scored better than at 14 days.

Bitterness

The varying bitterness seemed to have been caused by many factors in cheese and several researchers have previously reported on this. Fox *et al.* (1995) indicated that it was due to paracaseins which are normally tasteless but when degraded into by - products, peptides may be bitter, many amino acids have specific tastes, sweet and bitter. Also relative accumulation of low molecular weight hydrophobic peptides produce bitter cheese as reported by Visser *et al.* (1993) who found that their concentration remain below analytical threshold of detection. Level and specificity of proteolytic enzymes from lactic acid bacteria plays a critical role in determining whether or not bitterness develops. Report by Visser (1993) showed that the amount of rennet added in cheese is directly related to bitter flavour formation in cheese and this could apply to SUA Alpine cheese with 100HP and 100VPwhich were most bitter. The control cheese was also found to be bitter which could mean that other reactions in cheese could lead to bitterness. Steele, (1995) showed that dicarbonyls generated cheese flavour compounds including glyoxal and methylgloxal. The quality and type of dicarbonyls produced by lactic acid bacteria had a significant influence on the development of cheese flavour. This calls for more study on bitter compounds which were produced in these cheeses during ripening.

NaCl in most cheese was within the standard range but in some cheeses it might have been unfavourable for rennet induced hydrolysis of β-CN leading to a slow but gradual accumulation of bitterness, especially in Tilsiter 100CR, 100HP, 50VP and 75 VP which had a low NaCl content and low scores for bitterness. Fox and Stepaniak (1993) also

reported that C-terminal β-CN fragments such as β-CN (f193 - 209) and β-CN (f194 - 209) generated by the action of some lactococcal proteinases, contribute to bitter flavour development in cheese. The difficulty in identifying the main causative agent of bitterness in these cheeses has similarly been reported by many researchers (Walstra *et al.* 1993; Martely and Crow, 1993; Fox *et al.* 1995; Steele, 1995).

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 Experiment 1: Extraction of bovine pepsin

Optimisation of conditions for extraction of bovine pepsin from adult cattle abomasa

The basic conclusions from the study were:

- Good quality of pepsin could be obtained from sun dried fundic mucosa of cattle
- Extraction could be done in 10% NaCl at 25 - 30 °C
- Activation be done at pH 2 for 30 minutes
- Clarification be done with $Na_2HPO_4.H_2O$
- The extract contained 96 – 99% pepsin

Characteristics of bovine pepsin from different extraction solutions.

It was concluded that:

- Lemon juice was not suitable for bovine pepsin extraction.
- VP and HP extracts were as good as commercial rennet but VP should always be kept in liquid form in order to maintain its MCA.
- HP extract may be concentrated by freeze drying, direct sundrying and oven drying
- VP and HP were recommended for use in cheese making in Tanzania

Recommendation:Area of further research based on the present study.

There are some points which were not clearly defined and which may call for further studies. These are:

1. Investigation on storage conditions of extracts when NaCl concentration is increased to 18-20%
2. Clarification methods of low cost e.g. use of low heat
3. Peptides released during proteolysis on k-casein
4. Proportion of chymosin and pepsin in the extracts
5. Concentration methods of extracts and shelf life determination
6. Use of non electrical incubator for maintaining maceration temperature
7. Adoption of this method by small scale cheese processors
8. The level of purification desirable for commercial application under Tanzania conditions could be by use of $Na_2HPO_4.H_2O$.

6.2 Experiment 2: Purification of bovine pepsin

Purification of pepsin to homogeneity, demonstrated by SDS PAGE, required application of multi-step chromatographic techniques.

-HP was found to contain 96.5% pepsin and 3.5% chymosin while STD rennet contained 25% chymosin and 75% pepsin. The figures of STD rennet were similar to values which were given on the manufacturers label of 25% chymosin and 75% pepsin.

-As a result of purification, pepsin was resolved into three partially separated peaks on the Phenyl Sepharose which can represent the pepsin with different degree of

phosphorylation or pepsin from which the pepsinogen peptides with small differences in length had been cleaved

- Molecular mass of pepsin and chymosin were 34,000 and 34,500 Da, respectively by SDS PAGE and gel filtration.

Recommendations

Area for further research based on this study:

1. Investigate simple and cheaper methods of crude extract purification e.g. NaCling out

6.3 Experiment 3: Quality of cheese assesment of HP and VP extracts

This study has shown that:

- RT of cheese milk and TN loss in whey of CR/pepsin combinations were increasing with increasing level of pepsin. VP combinations gave longer RT than HP combinations and also lead to more loss of TN in whey especially 100VP. BF loss in whey was also high in 100VP.
- Chemical composition determinations varied a lot and were not directly related to the level of pepsin.
- Microbiological quality showed that cheeses had relative low counts for total count, yeasts / moulds when compared with the reported values. Low coliform counts in many cheeses indicated good hygienic quality.
- Sensory evaluation showed that all the cheeses had acceptable qualities except some bitterness and untypical taste noted in some SUA Alpine and Tilsiter cheeses.

- The indices used in quality assessment of cheese for chemical composition, microbial quality and sensory evaluation are obituary grades made from ranges found in literature or / and comparing with their respective cheese controls for each type of cheese. As summarized below the CR/pepsin combinations were not significantly different in all the types of cheese studied.
- Therefore all the combinations including 100% bovine pepsin were suitable for all types of cheese studied. But for best cheese perfomance: pepsin 75 HP for SUA Alpine, 50 VP for Village Alpine, 75 HP for Tilsiter, 50-75 VP for SUA Pasta filata and 50 VP for Village Pasta filata could be used to blend with CR.

Recommendations and areas of further research based on the present study

- It can be recommended that 50 to 75 % pepsin be used to blend with chymosin for Cheesemaking, but in case of chymosin shortage, 100% pepsin could still be used.

Areas which were not clearly defined and which may call for further studies include:

1. BF and TN losses in whey for the different CR/pepsin combinations
2. Flavour compounds produced by the enzyme combinations
3. Eletrophoretic studies of casein hydrolysis by enzyme combinations during cheese ripening
4. WSN expressed as percent total nitrogen (ripening index) of the cheese
5. Production of water soluble nitrogen during cheese ripening

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APPENDICES

Appendix 1. 1: Effect of ionic strength on milk clotting activity of extracts.

	pH levels of extracts			
Level of NaCl (%)	2	3	4	Mean
5	1089.03	2086.23	1826.13	1667.13^{c}
10	2124.10	2241.40	2202.00	2189.17^{a}
15	1558.70	1937.50	2034.45	1845.55^{b}
20	1021.45	2016.95	1757.90	1596.77^{c}
25	317.90	1630.25	1116.30	1021.48^{d}
Mean	1070.52^{a}	1726.45^{b}	1594.13^{c}	

Within each row and within column, means of PU/ml followed by the same later do not differ significantly at (P = 0.05) according to Duncan's multiple range test.

Appendix 1. 2: Effect extraction of pH and time (days) on milk clotting activity

Time	Level of pH						
(days)	1	2	3	4	5	6	Mean
1	1428.8	2792.2	3038.5	3810.5	2668.0	2971.4	2635^{d}
2	1678.4	3529.4	4040.4	2926.8	4622.2	3243.2	3340^{a}
3	2202.0	3753.7	5336.0	2581.0	3877.0	2857.1	3434^{a}
4	1548.5	2891.9	3582.9	3335.9	3693.2	2790.7	2973^{c}
5	2105.3	3428.6	3529.4	3428.6	3693.2	2666.7	3142^{ab}
6	1589.5	3818.2	3753.7	3750.0	4211.8	3200.6	3387^{a}
Mean	1759^{d}	3369^{b}	3880^{a}	3306^{b}	3794^{ab}	2805^{c}	

Within the row and each column means of PU/ml followed by the same latter do not differ significantly at (P = 0.05) according to Duncan's multiple.

Appendix 1.3: Effect of activation pH and time on MCA of the crude extracts

Analysis of valiance by general linear model procedure for activation pH and time

Source	DF	ANOVA SS	Mean square	F-Value	Pr>F
Reprication	1	162751.69	162751.69	0.79	0.3787 NS
Time	2	14549608.98	290921.79	14.18	0.000***
PH level	3	18803618.61	6267872.87	30.55	0.000***

	The level of pH for activation				
Time (sec)	1	2	3	4	Mean
10	2087.10	1128.00	1807.05	327.30	1337.4^{c}
20	2101.45	2474.45	3078.95	909.15	2141.0^{ab}
30	3333.30	3000.00	2526.60	1297.35	2539.3^{a}
40	3750.00	3001.85	2926.60	2000.00	2919.7^{a}
50	3693.20	3038.45	2400.00	2000.00	2783.1^{a}
60	3875.00	2891.95	2580.95	1983.60	2832.9^{a}
Mean	3140.00^{a}	2589.10^{b}	2553.4^{b}	1419.7^{c}	

Within each row and within the column, means of PU/ML followed by the same letter (s) do not differ significantly at (P = 0.05) according to Duncan's multiple range test.

Appendix 1. 4: Analysis of variance by general linear model procedure for effect of interaction between pH, temperature, NaCl and time on abomasal extraction

Source	Df	Sum of squares	Mean square	F value	Pr > F
Day	1	164738.6	164738.6	0.29	0.5940 NS
Temperature	2	3274676.1	16373338.1	2.84	0.0626 NS
NaCl	2	32586371.0	162931985.5	282.69	0.0001 ***
Ph	3	157294008.0	52431336.0	90.97	0.0001 ***
Acid	2	6541721.7	3270860.8	5.67	0.0045 **
NaCl*pH	6	38318094.5	6386349.1	11.08	0.0001 ***
Temp*NaCl	4	1904892.8	476223.2	0.83	0.5111 NS
Temp*pH	6	36409371.9	6068228.3	0.57	0.0001 ***
Day*NaCl	2	1848740.0	924370.3	1.60	0.2057 NS
Day*pH	3	7632035.0	2544011.7	4.41	0.0057 **

C.V = 30.66 Error = 72

** Significant at P< 0.01

*** Significant at P< 0.001

NS Non significant

Appendix 1. .5: Steps in the manufacture of Alpine cheese

Standardize 80 l milk to 3% butter fat (BF)
↓
Heat to 65°C / 30 min
↓
Cool to 35°C
↓
Add starter culture 2%
↓
Ripen milk for 30 min
↓
Inoculate CR/pepsin
↓
Incubate for normally 40 min to coagulate)
↓
Cut the coagulum into cubes
↓
Leave for 10 min to allow initial whey
↓
Remove 1/3 of whey
↓
Stir while cutting the large cubes
↓
Raise temperature to 38°C within 30 min while stirring using whey heated to 65°C
↓
Test the curd firmness and continue stirring at 38°C for another 30 min
↓
Drain the whey and divide the curd into four portions
↓
Press the curd into four cheese mould aligned with a cheese cloth 2 kg of cheese lever
↓
Press with 20 kg pressure / 2 kg cheese (10 kg pressure/ I kg cheese)
↓
After 1 hour turn the cheese and increase pressure 20kg cheese pressed overnight
↓
NaCl the cheese in brine solution of 15-20% NaCl for 12 hours
↓
Drill a 50g sample at the interval of 1,3,6 and 8 weeks.
↓
Seal the hole with melted wax.
↓
Cured at 18-19°C with 80% humidity for 6 weeks

overipening continued for 2 more weeks

Appendix 1. 6: Manufacture of Pasta cheese

Standardise 80 l milk to 3% fat

↓

Adjust temperature to 33-35^{0}C

↓

Add starter culture 2%

↓

Ripen for 15 min

↓

Add CR/pepsin

↓

Coagulate for 40 min

↓

Cut the curd into cubes

↓

Stir moderately for 15 min

↓

Raise the temperature to 38^{0}C by adding hot whey (65^{0}C)

↓

Left the curd to ripen for 2-4 hours

↓

Test the spinning ability of the curd

↓

When the curd is ready put in whey 85-90^{0}C

↓

Work the curds and put into the mould

↓

Cool in cold clean water

↓

Brine the cheese at 15% NaCl for 12 hr

↓

Cured and drill samples at 1, 2 and-4 weeks

and stored in the freeze for analysis

Appendix 1. 7: Manufacture of Tilsiter cheese

Mix half pasteurised evening and morning unpasteurized milk

↓

Raise temperature of milk to 25^0 C

↓

Evening milk serves as starter culture

↓

Add CR pepsin level

↓

Leave to coagulate for 40 min.

↓

Cut coagulum into cubes

↓

Stir cubes for 10-15 min

↓

Stir and scald with hot whey until the curds were plastic like

↓

Divide into 1kg portions

↓

Press with 10 kg for 1 hour

↓

Turn and leave over night

↓

Brine the cheese in 15% NaCl, label

↓

Leave to mature while drilling samples at 1, 3, 6 and 8 weeks interval.

Appedix 1. 8 : THE BALLOT FOR SENSORY EVALUATION OF CHEESES BY SCORING TEST

THE DEPARTMENT OF ANIMAL SCIENCE AND PRODUCTION
CHEESE TASTE PANNEL

No of candidate_____ Age___ Sex___ Race

Level of education: Primary__ Secondary__ BSc__MSc__PhD

Please look and taste each sample of cheese in order from left to right as shown on the ballot. Evaluate and score the samples of cheese for their sensory characteristics.

- For sensory characteristics 1-3 score as:
  Very good (3) Good (2) Poor (1)

- For sensory characteristics 4 score as:
  Not bitter (3) Slightily bitter (2) Bitter (1)

1. SMELL

$Code_1$ _____	$Code_4$ _____	$Code_7$ _____
$Code_2$ _____	$Code_5$ _____	$Code_8$ _____
$Code_3$ _____	$Code_6$ _____	$Code_9$ _____

2. TASTE

$Code_1$ _____	$Code_4$ _____	$Code_7$ _____
$Code_2$ _____	$Code_5$ _____	$Code_8$ _____
$Code_3$ _____	$Code_6$ _____	$Code_9$ _____

3. APPEARENCE AND CONSISTENCY

$Code_1$ _____	$Code_4$ _____	$Code_7$ _____
$Code_2$ _____	$Code_5$ _____	$Code_8$ _____
$Code_3$ _____	$Code_6$ _____	$Code_9$ _____

4. BITTERNESS

$Code_1$ _____	$Code_4$ _____	$Code_7$ _____
$Code_2$ _____	$Code_5$ _____	$Code_8$ _____
$Code_3$ _____	$Code_6$ _____	$Code_9$ _____

Comments: _____
_____

Appedix 1. 8 : THE BALLOT FOR SENSORY EVALUATION OF CHEESES BY SCORING TEST

THE DEPARTMENT OF ANIMAL SCIENCE AND PRODUCTION
CHEESE TASTE PANNEL

No of candidate_____ Age___ Sex___ Race

Level of education: Primary__ Secondary__ BSc__MSc__PhD

Please look and taste each sample of cheese in order from left to right as shown on the ballot. Evaluate and score the samples of cheese for their sensory characteristics.

- For sensory characteristics 1-3 score as:
  Very good (3) Good (2) Poor (1)

- For sensory characteristics 4 score as:
  Not bitter (3) Slightily bitter (2) Bitter (1)

1. SMELL

$Code_1$ _____	$Code_4$ _____	$Code_7$ _____
$Code_2$ _____	$Code_5$ _____	$Code_8$ _____
$Code_3$ _____	$Code_6$ _____	$Code_9$ _____

2. TASTE

$Code_1$ _____	$Code_4$ _____	$Code_7$ _____
$Code_2$ _____	$Code_5$ _____	$Code_8$ _____
$Code_3$ _____	$Code_6$ _____	$Code_9$ _____

3. APPEARENCE AND CONSISTENCY

$Code_1$ _____	$Code_4$ _____	$Code_7$ _____
$Code_2$ _____	$Code_5$ _____	$Code_8$ _____
$Code_3$ _____	$Code_6$ _____	$Code_9$ _____

4. BITTERNESS

$Code_1$ _____	$Code_4$ _____	$Code_7$ _____
$Code_2$ _____	$Code_5$ _____	$Code_8$ _____
$Code_3$ _____	$Code_6$ _____	$Code_9$ _____

Comments: _____

Appendix 1.9: ANOVA Table for chemical composition of different cheeses

Cheese	SV	DF	Variables								
			pH	Acid	BF	MC	TS	Salt	TN	WSN	RI
SUA Alpine	% pepsin	8	0.176^{***}	0.010^{***}	7.89^{***}	41.28^{***}	41.26	2.29^{***}	13.9^{NS}	0.19^{***}	142^{NS}
	Week	3	0.087^{***}	0.039^{***}	7.84^{***}	381.83^{***}	381.83	19.8^{***}	7.61^{NS}	1.04^{***}	2060^{***}
	% pepxwk	24	0.032^{***}	0.005^{***}	5.29^{***}	11.46^{***}	11.46	1.95^{***}	7^{NS}	0.12^{***}	91^{NS}
	Residue	36									
Village Alpine	% pepsin	8	0.055^{***}	0.006^{***}	29.0^{***}	26^{***}	26	2.48^{***}	10^{***}	0.17^{***}	164^{***}
	Week	3	1.140^{***}	0.106^{***}	154^{***}	611^{***}	611	1.41^{***}	3^{***}	1.613^{***}	1860^{***}
	% pepxwk	24	0.035^{***}	0.001^{***}	7^{***}	14^{***}	14	1.32^{***}	8^{***}	0.141^{***}	225^{***}
	Residue	36									
Tilsiter	% pepsin	8	0.126^{***}	0.011^{***}	20^{***}	62^{***}	62	1.16^{***}	4.6^{NS}	1.15^{***}	236^{***}
	Week	3	0525^{***}	0.128^{***}	139^{***}	319^{***}	319^{***}	7.49^{***}	1.7^{NS}	6.09^{***}	1809^{***}
	% pepxwk	24	0.073^{***}	0.005^{***}	18^{***}	16^{***}	16^{***}	0.57^{***}	6.8^{NS}	0.35^{***}	832^{***}
	Residue	36									
SUA Pasta	% pepsin	8	0.017^{***}	0.017^{***}	6.34^{***}	9.6^{***}	9.6^{***}	1.07^{***}	0.26^{***}	146^{***}	7.56^{***}
	Week	2	0.301^{***}	0.301^{***}	8.26^{***}	12.3^{***}	12.5^{***}	0.49^{***}	0.84^{***}	167^{***}	6.74^{***}
	% pepxwk	16	0.014^{***}	0.014^{***}	2^{***}	5.47^{***}	5.5^{***}	1.10^{***}	0.01^{***}	172^{***}	7.73^{***}
	Residue	27									
Village Pasta	% pepsin	8	0.057^{NS}	0.057^{***}	27.2^{***}	25.1^{***}	25^{***}	1.5^{***}	0.06^{***}	114^{***}	11.82^{***}
	Week	2	1.532^{***}	1.532^{***}	99.8^{***}	96.3^{***}	96.7^{***}	37.9^{***}	1.13^{***}	596^{***}	12.35^{***}
	% pepxwk	16	0.056^{*NS}	0.056^{***}	3.4^{***}	13.1^{***}	13.1^{***}	0.56^{***}	0.08^{***}	89^{***}	5.54^{***}
	Residue	27									

Key :

BF = butterfat **MC= moisture**

TN = total nitrogen **WSN= water soluble nitrogen**

RI= ripening index **TS = Total solids**

Appendix 1.10 ANOVA Table for sensory evaluation of cheese

Cheese	Source	DF	Taste	Smell	Texture	Bitterness
SUA Alpine	Treatment	8	1.835 ***	0.276 ns	1.147 *	4.355 ***
	Residual		0.582	0.648	0.720	0.60
	Totals	152				
	Penalist	16				
Village Alpine	Treatment	8	0.778 ns	0.252ns	1.389**	0.733 ns
	Residual		0.647	0.615	0.633	0.728
	Totals	161				
	Penalist	17				
Tilsiter	Treatment	8	0.460 ns	0.484 ns	0.687 ns	1.545 ***
	Residual		0.686	0.649	0.730	0.578
	Totals	170				
	penalist	18				
SUA Pasta	Treatment	8	0.517 ns	0.230 ns	1.541 **	0.920 *
	Residual		0.687	0.579	0.670	0.603
	Totals	134				
	penalist	14				
Village Pasta	Treatment	8	0.236 ns	0.653 ns	0.257 ns	0.234 ns
	Residual		0.714	0.615	0.661	0.679
	Totals	143				
	penalist	15				

Ns = not significant different (P>0.05) * = significant different (P<0.05) *** highly =significant different (P<0.001)

Appendix 1.11: Overall assessment of SUA Alpine cheeses

Para Meters	100CR	100HP	25HP	50HP	75HP	100VP	25VP	50VP	75VP
PH	3	3	3	3	3	3	3	3	3
Acid	3	1	3	3	2	3	3	2	1
Moisture	3	3	1	1	2	3	1	2	3
NaCl	3	3	3	3	3	3	3	1	3
BF	2	2	3	3	3	2	3	2	1
TN	3	3	3	1	1	2	1	3	1
WSN	3	2	1	2	3	3	1	3	1
RI	3	2	1	2	3	2	2	3	3
SPC*	3	1	3	1	3	3	2	1	3
Coli*	3	2	2	3	3	3	3	3	3
YM*	3	2	2	1	2	3	2	2	3
Taste	2	2	3	3	3	1	2	1	3
Smell	3	3	3	3	3	3	3	3	3
Texture	3	3	3	3	3	3	3	3	3
Bitter	1	1	3	3	3	1	2	2	3
Mean square	2.73 ±0.15a	2.27 ±0.18a	2.47 ±0.21a	2.33 ±0.23a	2.67 ±0.16a	2.53 ±0.19a	2.27 ±0.21a	2.27 ±0.21a	2.47 ±0.24a

N = 15, SD = 0.774, p value = 0.778, DF = 8, Totals 134

Key 1 = fair 2 = good 3 = very good
1* = high count 2* = average count 3* very low count

Subscript with the same letter in the same row of square means were not significant different (P>0.05) according to DMRT.

One way analysis of variance with Bonferroni test to analyse the differences between groups of SUA Alpine cheeses, showed no significant difference (P>0.05). When arranging the means from the highest to lowest the ranging was as follows:

1. 100CR **2**. 75HP **3**.100VP **4**. 25HP and 75VP **5**. 50 HP **6**. 100HP, 25VP, 50VP

Therefore all the CR/pepsin combinations were suitable for SUA Alpine cheese making.

Appendix 1.12: Overall assessment of Village Alpine cheeses

Para Meters	100CR	100HP	25HP	50HP	75HP	100VP	25VP	50VP	75VP
PH	3	2	1	2	2	3	3	3	3
Acid	3	2	2	2	1	2	2	1	1
Moisture	3	3	1	3	2	3	3	3	1
NaCl	1	3	3	3	3	3	2	2	2
BF	1	2	3	2	2	3	3	3	1
TN	3	3	1	2	2	1	1	3	2
WSN	3	2	2	2	3	1	1	2	3
RI	3	3	1	2	3	3	3	2	1
SPC*	1	2	3	3	3	2	2	3	2
Coli*	1	3	3	2	2	3	3	3	2
YM*	2	2	3	3	3	1	1	3	3
Taste	3	3	3	3	3	3	3	3	3
Smell	3	3	3	3	3	3	3	3	3
Texture	3	3	3	3	3	3	3	3	3
Bitter	3	3	3	3	3	3	3	3	3
Mean square	2.40 ±0.23a	2.60 ±0.13a	2.33 ±0.23a	2.53 ±0.13a	2.53 ±0.17a	2.47 ±0.22a	2.40 ±0.21a	2.67 ±0.16a	2.20 ±0.22a

N = 15, SD = 0.75, p value = 0.80, DF = 8, Totals 134

Key: 1 = fair 2 = good 3 = very good
1* = high count 2* = average count 3* very low count

Subscript with same letter in the same row of square means were not significant different (P>0.05 according to DMRT.

One way analysis of variance with Bonferroni test to analyse the differences between groups o village Alpine cheeses, showed no significant difference (P>0.05). When arranging the means from the highest to lowest the ranking was as follows:

1. 50VP **2.** 100HP **3.** 50HP,75HP **4.**100VP **5.** 100CR, 25VP **6.** 25HP **7** 75VP

All the CR/pepsin combinations were suitable for village Alpine cheesemaking

Appendix 1.13: Overall assessment of Tilsiter cheeses

Para Meters	100CR	100HP	25HP	50HP	75HP	100VP	25VP	50VP	75VP
PH	3	3	3	3	3	3	3	3	3
Acid	3	3	2	1	3	1	3	2	3
Moisture	3	3	2	1	2	2	3	1	3
NaCl	1	2	3	3	3	3	3	3	2
BF	3	3	2	3	2	1	2	3	3
TN	3	1	1	2	3	3	3	3	1
WSN	3	3	1	3	2	2	1	3	1
RI	3	3	1	3	2	2	1	3	1
SPC*	3	3	3	3	3	3	2	3	1
Coli*	3	3	3	3	3	3	3	2	1
YM*	1	3	3	3	3	3	3	3	3
Taste	3	3	3	3	3	3	3	3	3
Smell	3	3	3	3	3	3	3	3	3
Texture	3	3	3	3	3	3	3	3	3
Bitter	1	2	1	3	3	3	1	2	2
Mean square	2.47 ±0.23a	2.73 ±0.15a	2.27 ±0.22a	2.66 ±0.18a	2.63 ±0.12a	2.53 ±0.19a	2.47 ±0.21a	2.66 ±0.16a	2.20 ±0.24a

N = 15, SD = 0.762, p value = 0.447, DF = 8, Totals 134

Key 1 = fair 2 = good 3 = very good
1* = high count 2* = average count 3* very low count

Subscript with same letter in the same row of square means were not significant different (P>0.05) according to DMRT.

One way analysis of variance with Bonferroni test to analyse the differences between groups of Tilsiter cheeses, showed no significant difference (P>0.05). When arranging the means from the highest to lowest the ranking was as follows:

1. 100HP,75HP **2.** 50HP, 50VP **3.** 100VP **4.** 100CR, 25VP **5.** 75VP

All the CR/pepsin combinations were suitable for Tilsiter cheese making.

Appendix 1.14: Overall assessment of SUA Pastafilata cheeses

Para Meters	100CR	100HP	25HP	50HP	75HP	100VP	25VP	50VP	75VP
PH	3	3	3	3	3	3	3	3	3
Acid	3	3	2	3	2	3	2	3	3
Moisture	3	3	3	3	3	3	3	3	3
NaCl	3	1	1	3	3	1	2	3	2
BF	3	3	3	2	3	3	3	3	3
TN	3	3	3	3	3	3	3	3	3
WSN	3	2	3	1	3	3	3	3	3
RI	3	1	2	1	2	3	3	3	3
SPC*	3	1	3	2	3	3	1	2	3
Coli*	3	3	3	3	3	3	3	3	3
YM*	3	3	1	2	2	2	2	2	3
Taste	3	3	3	3	3	3	3	3	3
Smell	3	3	3	3	3	3	3	3	3
Texture	1	3	3	3	3	3	2	3	3
Bitter	2	3	3	3	2	2	1	3	2
Mean square	2.80 ±0.14a	2.53 ±0.21a	2.60 ±0.19a	2.53 ±0.19a	2.73 ±0.12a	2.73 ±0.15a	2.4 ±0.19a	2.87 ±0.10a	2.87 ±0.10a

N = 15, SD = 0.620, p value = 0.395, DF = 8, Totals 134

Key 1 = fair 2 = good 3 = very good
1* = high count 2* = average count 3* very low count

Subscript with same letter in the same row of square means were not significant different (P>0.05) according to DMRT.

One way analysis of variance with Bonferroni test to analyse the differences between groups of SUA Pastafilata cheeses, showed no significant difference (P>0.05). When arranging the means from the highest to lowest the ranking was as follows:

1. 50VP, 75VP **2**. 100CR **3**. 75HP, 100VP **4**. 25HP **5**. 100HP, 50HP **6**. 25VP

All the CR/pepsin combinations were suitable for SUA Pastafilata cheese making.

Appendix 1.15 : Overall assessment of Village Pastafilata cheeses

Para Meters	100CR	100HP	25HP	50HP	75HP	100VP	25VP	50VP	75VP
PH	3	3	3	3	3	3	3	3	3
Acid	3	3	3	3	3	2	2	2	2
Moisture	3	3	3	3	3	3	3	3	3
NaCl	1	1	2	2	3	3	1	3	3
BF	3	3	3	3	3	3	3	3	3
TN	3	1	3	3	2	3	3	2	3
WSN	3	3	3	3	3	2	3	1	2
RI	2	1	3	3	3	2	3	1	2
SPC*	3	3	3	2	2	2	1	3	2
Coli*	3	2	3	3	2	2	3	2	2
YM*	3	3	1	1	1	2	3	2	1
Taste	3	3	3	3	3	3	3	3	3
Smell	3	3	3	3	3	3	3	3	3
Texture	3	3	3	3	3	3	3	3	3
Bitter	3	3	3	3	3	3	3	3	3
Mean square	2.80 ±0.14a	2.53 ±0.21a	2.80 ±0.14a	2.73 ±0.15a	2.67 ±0.16a	2.60 ±0.13a	2.67 ±0.18a	2.47 ±0.19a	2.53 ±0.16a

N = 15, SD = 0.65, p value = 0.80, DF = 8, Totals 134

Key 1 = fair 2 = good 3 = very good
1* = high count 2* = average count 3* very low count
Subscript with similar letter in the same row of square means were not significant different (P>0.05) according to DMRT.

One way analysis of variance with Bonferroni test to analyse the differences between groups of village Pastafilata cheeses, showed no significant different (P>0.05). When arranging the means from the highest to lowest the ranking was as follows:

1. 100CR, 25HP **2.** 50HP **3.** 75HP, 50V **4.** 100VP **5.** 100HP, 75VP **6.** 50VP

All the CR/pepsin combinations were suitable for village Pastafilata cheese making.

Therefore it can be concluded that all the CR/pepsin combinations were found suitable for the five types of cheese which were investigated.