THE STUDY OF HEAT-STABLE PROTEASE: EFFECTS ON CHEDDAR CHEESE QUALITY AND ON THE TOTAL BACTERIAL COUNTS DURING RIPENING

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BY

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ABSTRACT

A heat-stable protease T25 secreted by ***Pseudomonas fluorescens*** from raw milk was used to accelerate the ripening process of Cheddar cheese. Its effects on the growth and activity of starter cultures used in Cheddar cheese manufacturing were also tested. The presence of bacteria protease (T25) exerted indirect influence on the growth of starter cultures. The addition of the protease to milk used for Cheddar cheese manufacturing caused differences in the growth patterns of bacteria during the first month of ripening. There was a trend in the lowering of percent total fat, protein, nitrogen, moisture content and yield (dry weight) of the cheese to which bacteria protease was added. The pH changes observed in the control cheese sample during aging were slightly higher than the sample containing the bacterial protease. A gradual degradation of α_{S1}-casein fraction was observed in the protease treated cheese during aging. Also, T25 protease gives better activity with α-casein as a substrate compared to other casein fractions. Addition of this bacterial protease (5 mg/L, 9.45 mg/L, 10 mg/L and 20 mg/L) to pasteurized milk prior to the addition of renneting agent (porcine pepsin) in the manufacture of Cheddar cheese by the conventional method, results in a product which is able to achieve higher intensity of cheddaring flavour ($P<0.05$) and higher preference score ($P<0.01$) within 6 months of aging when compared to that of the control cheese not containing the bacterial protease. No off-flavour or bitterness was detected in the protease treated cheese throughout

the ripening period. The texture of the cheeses remained fine during aging. The results of this study indicates that the protease T25 secreted by ***Pseudomonas fluorescens*** is a suitable agent to accelerate the ripening process of Cheddar cheese.

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List of Abbreviations

A	Absorbance
AOAC	Association of Official Analytical Chemists
CFU	Colony Forming Units
DISC-PAGE	Polyacrylamide gel electrophoresis
EU	Enzyme Unit
FAO	Food and Agricultural Organization
g	Grams
h	Hours
L	Liter
min	Minutes
MSM	Mineral Salt Medium
rpm	revolution per minute
TCA	Trichloroacetic acid
TEMED	Tetramethylenediamine
TSB	Trypticase Soy Broth

Chapter 1
Introduction

1.1. Historical

Dairy products are among the most popular foods and constitute an important item in man's diet. Milk production and dairy industries occupy a prominent position in agriculture in general and in food processing in particular. Dairy products as we know them today, e.g. buttermilk, cream, and cheeses, are produced with a high degree of know how and sophistication and with special attention and care for the flavour aspects of these products. Cheese, in particular is made in almost every country in one form or another, and in the developed world where annual cheese consumption is the highest.

There is no real knowledge of the origins of cheese or cheese making, but the earliest records of human activities refer to cows and milk. These may be found in Sanskrit writings of the Sumarians in 4000 BC, in Babylonian records of 2000 BC, and in the Vedic hymns (Chapman and Sharpe, 1982). There is also reference to cheese in biblical times. They are recorded in the Old Testament in the book of Job 10:10 (1520 BC), 1 Samuel 16:8 and 2 Samuel 16:29 (1170-1017 BC). But written history is scarce until the periods of the Greek and Roman Empires when various authors have left written evidence. The preparation of cheese probably dates back many centuries to the time when nomadic tribes of eastern Mediterranean countries carried milk of domesticated mammals in sacks, made from animal skins, or gourds, or in vessels such as stomachs or bladders. If kept warm the milk rapidly became sour and separated into curds and whey. If the whey was drained from the curds, the latter could be dried to form a firm, cheesy mass that could be eaten fresh, or stored and eaten over long periods. In this way much of the food value of milk could be preserved for use when supplies of liquid milk were not available..

In time it was found that the secretion from the stomach of a young ruminant had the power to coagulate milk, which also reduced the time required to drain the whey from the curds. This eventually led to the use of rennet, an extracted enzymic secretion from the fourth stomach of a young calf, lamb or kid, to bring about the coagulation of milk, which is the first step in the process of cheesemaking as it is practised today. The cheesemaking process described by De Re Rustica in 50 AD shows that there had been a gradual evolution from the acidic curdling of milk by natural fermentation to the controlled production of a form of curd which could be preserved (Columella, 1945). This knowledge spread through the countries of the Roman Empire. In Britain there is no positive evidence of cheesemaking before the Roman conquest, though presumably some form of milk curds were used. During the ensuing years of Roman occupation, however, cheese became a well known food. Palladius, who wrote a treatise on "Agriculture in Britain" 300 years after the conquest advocated that cheesemaking should take place in early summer, that milk should be curdled with rennet obtained from the stomachs of the kid, the lamb or the calf or, alternatively, by the milk from the fig tree or teasel flowers. This is the first direct evidence of the coagulation of milk by agents other than natural acidity, and by rennets of vegetable as well as animal origin (Chapman and Sharpe, 1982).

It was not until the beginning of the present century that cheesemakers developed the modern practice of using carefully selected pure strains of the bacteria, comprising the "starter" cultures, which were deliberately added to cheese milk in standard amounts depending on the type of cheese required.

Different ways of making cheese were developed in different countries, and in different areas within a country, as the outcome of experience and to suit local and market demands. These cheeses, whatever the country of origin, were the forerunners of varieties which have been stabilized and named, and have assumed local, national and often international importance.

1.2. Types of Cheese

Whilst there are over 400 varieties of cheese, there are only about 18 distinctly different types. Many varieties are named after their place of origin and differ from one another only in shape and method of packaging; their method of manufacture and general characteristics being very similar. All varieties of natural cheeses are made from milk. They can be divided into three main classes, viz., soft, blue-veined and hard-pressed cheese. They vary widely in moisture content and, therefore, in keeping quality and method of ripening. The characteristics of a particular cheese variety are governed not only by the composition of the starter culture, but also by the temperature of manufacture, the coagulant used to gel the milk (chymosin or a microbial substitute) and by the secondary microflora which may be present as chance contaminants (e.g. non-starter lactic-acid bacteria) or introduced into the cheesemaking process deliberately (e.g. spores of ***Penicillium*** species).

Soft cheese curd retains a high proportion of moisture (whey) (55-80%). Some varieties are eaten fresh (Cambridge, Coulommier, Bondon, etc), whilst others are ripened, usually by the growth of surface moulds (Brie, Camembert, Pont l'Eveque, etc.). Semi-soft cheeses, such as Limburger, Tilsit and Brie, are made from slightly firmer curds (45-55% moisture) and are ripened by the surface growth of micro-organisms, particularly ***Brevibacterium linens***. These are the smear-ripened cheeses.

Blue-veined cheeses, such as Stilton, Roquefort and Gorgonzola, are made from semi-soft/semi-hard curd with 42-52% moisture, and are ripened by species of ***Penicillium*** moulds which grow within the cheese.

The semi-hard cheese, such as Edam and Gouda, are made from firmer curd with a moisture content within the range of 45-50%. The cheeses are ripened by bacteria and are consumed within 2-3 months.

The hard-pressed cheese are made from firm, relatively dry curd (35-45% moisture). They are ripened by bacteria and mature slowly over a period of 12 months. In some varieties such as Cheddar cheese made from calf-rennet, the aging time can be as long as 1-3 years. Acid is developed in the curd of Cheddar and Cheshire cheeses before they are salted and pressed. In other varieties (e.g.

Emmenthal and Gruyere) acid is developed while the curd is draining and being pressed, but before it is salted.

The very hard, grating cheeses, such as Parmesan, Romano and Asia[illegible] made from very firm curd. They are low-moisture cheeses (26-34%), made [illegible] partly skimmed milk and are ripened by bacteria, slowly over a period of 1-2 years.

The consistency of a cheese, its firmness or body, is determined by certain basic factors, and control of these is essential to ensure that the properties are characteristic of the variety and will provide suitable conditions for correct ripening. Softness is favoured by high moisture content, high fat content and extensive proteolysis. The opposite of these features characterise the hard varieties of cheese with firm body.

The variety of cheese to be produced in any class is determined by the type of milk used, the preparation of the young curds, and the inclusion in the milk or curds of certain micro-organisms responsible for the development of acidity during manufacture, and the development of characteristic features and flavours during ripening. The types of bacteria or moulds which, by their growth during cheesemaking or cheese-ripening, participate in the process, are determined by deliberate inoculation of specific organisms, conditions of cheesemaking and environmental factors.

1.3. World Production of Cheese

Cheese and fermented milks are among nature's most important contributions to civilization. Historically, these foods have enabled populations to survive periods of famine; nutritionally, they provide elements vital to good health, making them desirable staples in man's daily diet; and geographically, they lend themselves well to realistic production in many developing countries. Cheese has even been used as a form of currency. It is usually an indispensible item in mountain climbers' knapsacks; and in Switzerland, the Saanen type is held for years to commemorate anniversaries, births and weddings (Kosikowski, 1978).

Cheese production has increased by four million tonnes from 1961-76. This is equal to an annual increase of 4.4 % (Scott, 1981). According to the information from FAO Yearbook (1984), total world production of cheese is 12.4 million metric tonnes. Table 1-1 shows the production of cheese in various areas of the world.

1.4. Nutritional Advantage of Cheese

Cheese comes in all sizes and shapes and have different names. Cheddar cheese, one of the most popular cheese is also one of the cheapest sources of high protein value (Scott 1981). Cheddar cheese originated many decades ago in the little village of Cheddar, England from which it spread throughout the world. The increase in total world cheese production can be attributed to the nutritional advantage of cheese over other everyday foods.

Table 1-1: WORLD PRODUCTION OF CHEESE (ALL KINDS) IN 1984

Area of the World	Production (Metric tonnes)	Countries included and their production in (MT)	
Africa	378,123	Egypt	244,250
North America	2,631,415	USA	2,402,000
		Canada	229,415
South America	423,701	Argentina	210,000
		Brazil	59,150
Asia	674,684	China	123,479
		Japan	70,000
Oceania	275,000	Australia	160,000
		New Zealand	115,000
Europe	6,122,200	UK	245,000
		France	1,250,000
USSR	1,659,000		

Total World Production (1984) = 12.4 million metric tonnes

MT = Metric tonnes

Source: FAO Production Yearbook, Vol. 38, 1984. Food and Agricultural Organization of the United Nations (Rome 1984).

1.5. Ripening of Cheese

Ripening of cheese involves changes in the chemical and physical properties of the cheese accompanied by the development of characteristic flavour. Different varieties of cheeses have different methods of ripening (Kosikowski, 1985). Table 1-3 indicates different methods of ripening of various types of cheese.

Fresh, young cheese curd is tough and, sometimes rubbery. It consists mainly of protein, fat and moisture, in varying proportions depending on the type of cheese, together with small amounts of salt, lactose, lactic acid, whey proteins and minerals. In ripening, this curd is gradually digested by enzymes, and the mature cheese acquires the firm, or plastic, or soft body characteristic of the particular variety. The chemical changes responsible for ripening cheese are: (1) fermentation of lactose to lactic acid, small amounts of acetic and propionic acid, carbon dioxide and diacetyl, (2) proteolysis, and (3) lipolysis. These changes are brought about by enzymes from (i) the lactic acid bacteria of the starter culture, (ii) non-starter bacteria in the milk, (iii) the rennet, rennet paste or rennet substitute used to coagulate the milk, (iv) the milk itself, and (v) other micro-organisms growing within or on the surface of the cheese. These metabolic changes are accompanied by the development of characteristic flavour. They are affected by the size and composition of the young cheese, and are controlled by the conditions of temperature and humidity at which the cheese is ripened and stored. Block stacking of warm cheese on pallets and block stacking of pallets can influence temperature and flavour differences between blocks of cheese from the same making vat (Miah *et al.*, 1974). Some varietes (e.g. Emmenthal, Camembert

Table 1-2: METHOD OF RIPENING OF DIFFERENT VARIETIES OF CHEESE

CHEESE	MILK	METHOD OF RIPENING
		HARD CHEESE
Asiago	Cow or Ewe	Cured for up to a year washed and turned frequently and sometimes rubbed with vegetable oil.
Cheddar	Cow	Cured at 36-50°F for 60 days to 12 months.
Colby	Cow	Cured for 60 days or more.
Edam	Cow	Shelved in layers at 50-60°F for 6 to 8 weeks. Washed, dried and turned frequently.
Emmentaler (Swiss)	Cow	Formation of eyes in 3 to 4 weeks at 72°F and 80-85% relative humidity. Ripened at 40°F and higher temperatures for 2 to 10 months.
Gouda	Cow	Cured at 50-60°F for 2 to 6 months.
Gruyere	Cow	Formation of eyes at 60°F in 1 month. Cured for 80 days or more at 50-60°F.
Parmesan	Cow	Shelved for 10 months or more at about 50°F and 85% relative humidity. Turned, and washed, scraped and rubbed with oil from time to time.
Provolone	cow	smoked and cured at 40-50°F for up to 12 months.
Stilton	Cow	Mold-ripened by *Penicillium roqueforti* for 2 weeks, cured for about 6 months.

Table 1-3 Con'd

CHEESE	MILK	METHOD OF RIPENING
		SEMISOFT CHEESE
Bleu (Blue)	Goat or Cow	Mold-ripened by *P. roqueforti*, cured for 3 months at 48°F and 95% relative humidity, wrapped in foil and stored in a cool room for 2 to 3 months.
Brick	Cow	Cured on surface by ***Bacterium linens*** for 14 days, wrapped and stored for 2 to 3 months at 40°F.
Gorgonzola	Cow	Mold-ripened by *P. roqueforti*, cured at 40-50°F and 80% relative humidity for 30 days, then at higher humidity for 3 to 6 months.
Monterey	Cow	Cured for 6 weeks or more at 60°F and 80% relative humidity.
Muenster	Cow	Cured for several weeks at 50-55°F and 80% relative humidity.
Roquefort	Ewe	Mold-ripened by *P. roqueforti*. Held Wheels are salted and stored in caves at Roquefort at low temperature and high relative humidity for 3 months.

Table 1-3 Con'd

CHEESE	MILK	METHOD OF RIPENING
		SOFT CHEESES
Brie	Cow	Ripened by a white mold, ***P. candidum***, for 8 to 11 days in cellar or cave at 52°F and 90% relative humidity. Distributed within 14 days under refrigeration.
Camembert	Cow	Ripened by ***P. candidum***, on frames or shelves at 55°F and about 95% relative humidity for 12 days. Distributed within 21 days under refrigeration.
Liederkranz	Cow	Ripened by ***B. linens*** for 3 to 4 weeks at 45°F.
Limburger	Cow	Ripened on surface by ***B. linens*** and cured on shelves for 3 weeks at about 55°F and 95% relative humidity.

and Stilton) require special periods of controlled temperature and humidity for the ripening process, during which bacterial or fungal activity produces specific changes in the body, texture and flavour of the cheese. Ripening is then followed by storage until the cheese is ready for sale. Other varieties, particularly the hard cheeses without eyes (e.g. Cheddar and Parmesan) are stored at constant temperature throughout the ripening period, and maturation may extend over many months.

During ripening, characteristic changes take place in the body, texture and flavour of the cheese. The term "body" is used to describe the consistency of cheese, and includes such attributes as firmness, elasticity, plasticity and cohesiveness. Texture describes the structure or presence of "holes" within the cheese. Development of characteristic flavour and aroma compounds during the process of ripening are caused by the action of micro-organisms and enzymes which break down proteins, fats and carbohydrates and, in some cases, metabolize lactic acid, lactate and citrate.

The changes first occur in a crude way: the original curd, with a coarse structure and a different degree of dispersion, changes into a more or less plastic, homogeneous substance, often including holes; the structure is either uniform or shows a stepwise ripening indicated by different layers. On the surface, we mostly find a developing rind, and often a growth of mold cultures is seen either on the surface or inside the cheese substance.

In addition to visible properties, there are chemical changes taking place.

At first they are noticeable by the evolution of a characteristic odour for each cheese, which varies in intensity and is often highly esteemed by the consumer. Among the three components of the cheese substance - protein, carbohydrates and fat, the protein is often decomposed significantly. The main change is in the casein component, which is separated from milk either with rennet or acid. Decomposition of the carbohydrate component, i.e. lactose, also plays an important role in the ripening process of cheese. Decomposition of fat is of particular significance in mold-ripened cheese.

The chemical changes are usually catalyzed by the enzymes formed by the microorganisms involved in ripening, and far less often by enzymes which are derived from the milk. The main causes of cheese ripening, are microbiologic- and enzyme-induced changes. Among the ripening reactions are the decomposition or synthesis of a wide variety of substances, such as proteins, peptides, amino acids, carbohydrates, lipids, nucleic acids, organic acids, various carbonyl compounds, growth factors from the groups of vitamins, prosthetic groups of enzymes, and, finally, simple decomposition products, such as carbon dioxide and ammonia. Various products of protein hydrolysis, as well as fatty acids and their esters or ketones, may be present in varying amounts in the cheese. This produces a complex mixture of components which give the required balance of flavour (Fryer, 1969) characteristic for the variety. The starter bacteria die out during ripening, as do most other organisms present in the curd, including enterococci and leuconostocs. Only the lactobacilli, which may be present in fresh curd in small numbers, multiply, and these may reach levels of 10^6 to 10^8 g^{-1} in cheese in 3-6 weeks (Sharpe, 1979).

1.6. Present Problems Associated With Cheesemaking

Until recently, calf rennet has been the traditional coagulant used in cheese manufacture. Cheddar cheese prepared with an enzyme extract from the young calf produces a high quality product after a long aging time of 1-3 years at a temperature of 10°C. The aged product is referred to as "old", "very old" or "sharp" Cheddar. It commands a higher price than freshly sold or "mild" Cheddar. The lengthy aging times are an impediment to the cost effective production of "old" Cheddar cheese.

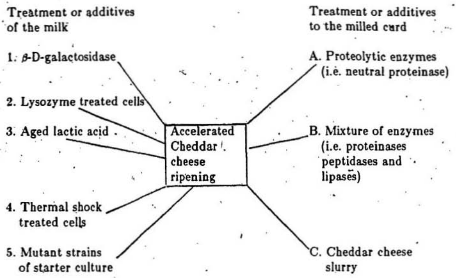
In addition, a decline in the number of calves slaughtered nowadays and an increase in world-wide cheese production has resulted in a short supply of calf-rennet (Cheeseman, 1981). These factors have led to an active search for suitable substitutes offering a comparative price advantage over the traditional calf-rennet coagulant (deKoning, 1978). Today, the majority of cheese made in the world is prepared with alternative enzyme known as "rennet substitutes". The commonly used rennet substitutes are porcine pepsin, bovine pepsin, and proteases from *Mucor miehei*. In 1974, two-thirds of the cheese manufacturing in the United States utilized rennet substitutes obtained from sources such as *Mucor miehei, Endothia parasitica* or *Mucor pusilus* (Huang and Dooley, 1976). Rennet substitutes differ from calf rennet in substrate specificity. As a consequence, more intense or less specific breakdown in the cheese casein may occur causing low yield of curd and defects in the body and flavour of the finished product. Another disadvantage of commonly used rennet substitutes is that they do not facilitate the aging process in Cheddar cheese manufacture as effectively as calf rennet. As a result, they are commonly employed for the production of "mild" Cheddar cheese. In the specific case where porcine pepsin is employed as a rennet substitute in Cheddar cheese manufacture, it is known that the enzyme is unstable at the pH and temperatures employed during the "Cheddaring" stage of the process. As a result, Cheddar cheese prepared using porcine pepsin as a renneting agent ages very slowly compared to the Cheddar cheese prepared with calf-rennet..

Cheese ripening is a complex process brought about by enzyme or enzyme systems provided by bacteria, moulds and yeasts, changing gradually the fresh rubbery curd to a mellow waxy product having characteristic flavour and aroma. The aging of Cheddar cheese is affected by the type of coagulant employed in the renneting step of cheese making. Generally, the more expensive calf-rennet is employed for the preparation of "old" Cheddar cheese because the resulting product is of good quality. Aging of Cheddar cheese is also facilitated by the use of raw (not pasteurized) milk or of partially pasteurized milk. The maturation of Cheddar cheese is also influenced by the temperature of aging. Most of the hard varieties of cheese like Cheddar cheese require a long period of maturation. For this reason significant cost goes to provide time for ripening and storage. The investment by cheese industry in storage time during ripening of only Cheddar cheese is equivalent to about 19.5 million dollars a month (Jamil Ud Din Warsy, 1983). It was estimated in early 1976 that the aging cost was 1.3 cents per pound of cheese per month (Jamil Ud Din Warsy, 1983). Since then, due to inflation and energy costs, the cost for cheese production has undoubtedly increased.

In order to alleviate this problem, many investigators in different research laboratories have attempted to accelerate the ripening process of Cheddar cheese. Figure 1-1 illustrates different methods used for accelerating the ripening of Cheadar cheese (Ridha *et al.*, 1984a).

Figure 1-1 summarizes the different methods used to accelerate the ripening process of Cheddar cheese. Some of these methods employed include: the addition of β-D-galactosidase to milk (Marschke and Dulley, 1978; Ridha, *et al.*, 1983 and 1984b), the addition of specially treated cells of mesophilic lactic acid bacteria (Dulley *et al.*, 1978), the addition of Cheddar cheese slurry - and/or the addition of protease and lipases (Law, 1978). The primary objective of the former three methods was to increase the number of micro-organisms and their enzymes in cheese with the aim of accelerating the flavour development in Cheddar cheese. The latter method involves the addition of enzymes from animal or microbial sources. Nowadays, a limited number of products are available commercially as cheese ripening aid, e.g. β-galactosidase (Maxilact™, G. B. Fermentations). The microbial proteases used to accelerate the ripening process are neutral protease of ***Bacillus subtilus***, acid protease from ***Aspergillus orryzae***, alkaline proteases from ***B. licheniformis*** (Law and Wigmore, 1982), and proteases from ***Kluyveromyces lactis*** (Grieve *et al.*, 1983). The bacterial mutants used include ***Streptococcus lactis*** lac⁻ mutants (Dulley *et al.*, 1978).

FIGURE 1-1: DIFFERENT METHODS USED FOR ACCELERATING THE RIPENING OF CHEDDAR CHEESE



1.7. Limitations or Drawbacks of Present Process

The disadvantage of using raw milk is that undesirable micro-organisms (e.g. pathogens like *Salmonella* species or spoilage organisms) may be present. Hence, the cheese manufacturer does not have the desired control over the final quality of the product and for this reason either the practice of using raw milk for cheesemaking is discouraged or banned by regulatory agencies in most provinces.

Increased aging temperatures may adversely affect the normal flavour balance due to overproduction of some classes of compounds e.g. free fatty acids, peptides and sulphur compounds. High temperatures may encourage the proliferation of unwanted and spoilage organisms (Law, 1981). The use of exogenous proteinases, though they give strong flavours in cheese in relative short time, they often induce flavour defects and flavour imbalance (Law, 1983). Commercial proteases having a high ratio of endo-to exo- peptidase activity tend to cause excessive gross proteolysis leading to abnormal body or texture development. Some may even cause bitter off-flavour in aged cheese.

The efficacy of using β-galactosidase (lactase) to enhance the flavour development in Cheddar cheese has been subjected to critical scutiny. This is partly due to the lack of definitive evidence as to the relative efficiency with which cheese microorganisms utilize lactose and glucose and galactose (Law, 1984). It has been shown that commercial lactase used to accelerate cheese ripening contain contaminating proteolytic enzymes (Law and Wigmore, 1983).

Most attempts to accelerate typical flavour development in Cheddar cheese have been impeded by the difficulty of maintaining a flavour balance. Some of the problems were apparent from the incidence of rancidity and bitterness. In 1975, Kosikowski and Iwasaki used the mixtures of commercially available preparations of fungal rennets, neutral and acid proteinases, peptidases and lipases to produce strong flavours in cheese in one month, but rancidity was particularly noticeable. Their preliminary finding indicated that fungal acid proteinases and fungal decarboxylases did not contribute to cheese flavour.

The efficacy of lipases as agents for rapid ripening of Cheddar and related types is open to interpretation, perhaps because the notion of "typical mature flavour" has changed as this type of cheese has been made and consumed in more and more countries. However, attempts to accelerate the development of typical flavour in English Cheddar cheese using commercial lipases have failed (Law, 1984); the lipases were screened for their ability to release either short- or long-chain fatty acids in order to differentiate between their effects on flavour. The long-chain (C_{12}-C_{16}) fatty acids released by a ***Mucor meihei*** lipase produced an unpleasant "soapy' flavour defect, while the short-chain acids released by animal esterases produced an unclean flavour. Many levels of addition were investigated but these enzymes either produced no flavour effect at all or they produced defects; no compromise could be reached whereby desirable flavour could be enhanced without defects. Even when they were added together with proteinases, the lipase did not accelerate the formation of typical flavour (Law, 1984).

1.8. Objective of This Study

The presence and metabolic activity of psychrotrophic microorganisms in milk and dairy products affect the quality of finished product (Stepaniak *et al.*, 1982; Law *et al.*, 1979; Cousin, 1982). It is hypothesized that the addition of heat-stable protease to pasteurized milk prior to the addition of a renneting agent (e.g. calf rennet or porcine pepsin) can result in a Cheddar cheese which matures faster than the control cheeses (not containing the added microbial protease). In this study, an attempt was made to test this hypothesis. Porcine pepsin was used as a renneting agent instead of traditional calf rennet because from the preliminary study of using a commercial calf rennet as a renneting agent, it tends to give rise to bitter taste in Cheddar cheese whereas when porcine pepsin was used as a renneting agent, bitter taste was not detected. The reason is that the commercial calf rennet was not 100% pure calf rennet. Moreover, the following aspects of the heat-stable protease were also investigated:-

1. The effect of the heat-stable protease on the total and free amino acids in the Cheddar cheese during the process of ripening.

2. The effect of the protease on total bacterial counts during the ripening of the Cheddar cheese.

3. The effect of the protease on the growth of the starter cultures grown in synthetic medium.

4. The effect of the protease on the breakdown of caseins in ripening of Cheddar cheese.

5. The effect of the protease on the composition of the Cheddar cheese was examined specially with respect to total lipids (fat), protein, moisture and total nitrogen content.

Chapter 2

The effect of heat-stable protease from psychrotrophic pseudomonad (Pseudomonas fluorescens T25), on the ripening process of Cheddar cheese

INTRODUCTION

Cheddar cheese prepared with an enzyme extracted from young calf "calf-rennet" produces a high quality product but it also requires a long aging time of 1-3 years. A significant proportion of the operating costs go to provide space for Cheddar cheese ripening. The storage time required is longer for cheeses with long ripening times. Cheese manufacture is now a capital-intensive industry which benefits from a high rate of turnover and the running costs and interest charges involved in cheese storage represent a significant proportion of the total cost of converting milk into cheese (Law and Wigmore, 1982). Therefore, the lengthy aging time is an impediment to the cost effective production of "old" Cheddar cheese. Any shortening of the time cheese is kept in store therefore represents a worthwhile saving provided that flavour development can be accelerated without impairment of flavour balance. In addition, a decline in the number of calves slaughtered nowadays and an increase in world-wide cheese production tend to aggravate the problems faced by dairy industry. Raising the storage temperature of the cheese is the most obvious method but while this may speed up flavour-forming reactions, it also speeds up off-flavour formation and may promote the growth of unwanted microbial contaminants such as moulds; selective methods of accelerating ripening of cheese are therefore required. These factors have led to an active search for suitable substitutes offering a comparative price advantage over the traditional calf-rennet coagulant.

Raw milk from different locations may contain psychrotrophic bacteria capable of producing heat-stable proteases (Adams *et al.*, 1975; Barach *et al.*, 1976; Richardson and TeWhaiti, 1978; Griffiths *et. al.*, 1981; Marshall and Marstiller, 1981; Stepaniak *et al.*, 1982; Kraft and Rey, 1979). The presence of these heat-stable proteases in milk may have direct or indirect effects on the subsequent quality of dairy products especially cheese. The influence of these heat-stable proteases on various aspects of cheese-making is little understood at the present time. Hick *et al.* (1982) and Law *et al.*, 1979 have studied the influence of psychrotrophic bacteria on the yield and quality during the preparation of Cheddar cheese. In an attempt to cut down the high cost of cheese production faced by the dairy industry by shortening the aging time and without affecting the quality of Cheddar cheese, a type of heat-stable protease from psychrotrophic pseudomonad (***Pseudomonas fluorescens*** T25) was used in the present study. This type of microbial protease is easy to prepare because it is secreted by the organism in the medium supporting its growth.

MATERIALS AND METHODS

2.1. MATERIALS

Samples of raw milk were purchased from a local milk plant (Kelsey J & Sons Ltd). Most of the bacteriological media and reagents were purchased from Difco Laboratories (Detroit, MI); all other chemicals were of analytical grade and were purchased either from Sigma Chemical Co. (St. Louis, MO) or from British Drug House Ltd.

2.2. METHODS

2.2.1. Source of Microbial Protease

A heat-stable protease of psychrotrophic origin was used to accelerate the ripening of Cheddar cheese. The strain of the bacterium used was ***Pseudomonas fluorescens*** (T25) and was isolated from raw milk by the method described earlier by Patel ***et al*** (1983a).

2.2.2. Enzyme Preparation

P. fluorescens (T25) was grown in Trypticase Soy Broth (TSB) (BBL Microbiology Systems, Cockeysville, Md.) containing 1-2% skim milk powder incubated at 25°C for 4 to 5 days on a shaker (Psychrotherm, New Brunswick Scientific Co., New Brunswick, NJ). For maximum enzyme production, the culture (0.1 to 0.2%, vol/vol) was inoculated into several 500 mL Erlenmeyer flasks, each containing 125 mL of sterile medium. Cells were removed by centrifugation at 8000 x g for 15 min in a centrifuge (Ivan Sorvall Inc., Norwalk, Conn.). The clear supernatant solution was decanted and extensively dialyzed in

20 mM Tris-HCl buffer, (pH 7.2). The dialyzed extract was the source of the protease. When necessary, the crude cell extact was concentrated by lyophilization. The dry residue obtained after lyophilization was redissolved in a minimum quantity of the same buffer, and was dialyzed against the Tris-HCl buffer. Freezing and thawing of the crude enzyme preparation had no adverse effect on the protease activity.

2.2.3. Protease Assays

The protease activity was determined by modified Hull's method (Patel *et al.*, 1983). The substrate, soluble casein, and enzyme samples were extensively dialyzed in 0.1M of Tris-HCl buffer, at pH 7.5 before use. The reaction mixture contained the following (in a total volume of 2 mL): 1.5 mL of Tris-HCl buffer (100 mM, pH 7.5); 0.2 to 0.4 mg of enzyme protein; and 0.5 mL of substrate (1% soluble casein solution). The reaction mixture was incubated at 25°C for 10 to 30 min in a temperature-regulated water bath. The reaction was stopped by adding 1.0 mL of 5% trichloroacetic acid solution. The precipitated proteins were removed by centrifugation, and the trichloroacetic acid-soluble free aromatic amino acids in the clear supernatant solution were estimated by absorbance at 280 nm. Tubes containing either substrate and no enzyme or enzyme but no substrate were included as controls. One enzyme unit (EU) is the amount of extract that releases 1 μmol tyrosine equivalent per min per ml at 25°C. Specific activity is enzyme units per mg of protein. The specific activity of microbial protease (T25) was about 0.15 EU/mg of protein.

2.2.4. Cheddar Cheese Making

The Cheddar cheese was made by the conventional method as described by Kosikowski 1978a with some modifications. The process entails the following steps:

1. Preparing the starter culture
2. Pasteurization of milk
3. Adding of Protease
4. Adding of starter culture
5. Forming the curd (Adding of porcine pepsin)
6. Cutting the curd
7. Cooking the curd
8. Draining
9. Cheddaring
10. Milling
11. Salting
12. Pressing the curd
13. Ripening the young cheese

Two batches of cheese were made in the laboratory. One with the addition of microbial protease (9.45 mg/L), and the other was a control (i.e. without adding bacterial protease). Duplicate experiment was repeated 3 months later. Chemical analysis and sensory evaluation were carried out on both batches of cheese at their respectively ripening period of 3, 6 and 9 months.

2.2.4.1. Preparing the Starter Culture

One litre of homogenized whole milk was heated to a temperature of 88°C. It was held at this temperature for 30 min. The milk was cooled to room temperature. One per cent (10g/L) of the commercial frozen "starter" ("Superstart" concentrated culture, Marschall Products, Miles Lab. Madison, USA) containing a mixture of *Streptococcus lactis* and *S. cremoris* was added. It was left at room temperature overnight to allow the culture to grow and set the milk.

2.2.4.2. Pasteurization of Milk

As a rule, the milk for making a ripened cheese is raw or partially pasteurized. Fully pasteurized milk also serves, but the partially pasteurized kind is the commoner choice. It appears that the enzymes from the micro-organisms that survive the lower temperature give rise to a better flavour in the cheese.

Sixteen liter batch of raw milk, purchased from a local milk plant was heated to a temperature of 63°C. It was held at this temperature for 30 min. This process is known as pasteurization. It has the objective of killing the pathogens that are responsible for infectious diseases. The milk was then cooled to 30°C.

2.2.4.3. Addition of Heat-Stable Microbial Protease

Heat-stable protease (T25) was added to the milk after it had cooled down to 30°C. The concentration of the added enzyme was 9.45 mg/L. For the control cheese, this step was omitted.

2.2.4.4. Addition of Starter Culture

The pH of the starter culture was recorded. The starter culture (80 g/16L) was added, with constant stirring, to the pasteurized milk (16 L) After the addition of the starter culture, pH of the milk was recorded at a time interval of 30 min until a pH drop of 0.03-0.05 was observed. The final pH was 6.4.

2.2.4.5. Addition of Clotting Enzyme, Porcine Pepsin

Milk was transformed into a smooth, solid curd by the addition of a coagulating enzyme. The enzyme used was porcine pepsin (1:10,000 Sigma Company).. A concentration of 10 mg/mL of the coagulating enzyme was used. The porcine pepsin which was in powder form was first dissolved in 20 mM of acetate buffer at a pH of 5.3. The volume of the enzyme added was 45 mL/16 L. It was added to the milk while stirring in order to prevent localized coagulation. After the addition of porcine pepsin, the milk was left undisturbed for 30 min at 30°C.

2.2.4.6. Cutting The Curd

At the end of 30 min sterilized cutting wire knives were used to cut the large bed of curd in the cheese vat horizontally and then vertically into cubes about 1.5 cm on a side. This step increases the surface area. The purpose of cutting or breaking the curd is to speed whey expulsion and assist in uniform cook-through of the curd by increasing the surface area.

2.2.4.7. Cooking The Curds

The curd, when first cut, was soft and the coat surrounding the particles was open. Stirring the curd gently until the first flush of whey has left the curd particles was necessary to prevent undue crushing and loss of fat and curd dust.

The temperature of the curd was increased gradually by 1°C in every 5 min to a final temperature of 38-39°C. The curd was stirred occassionally. Scalding or cooking the curd causes the protein matrix to shrink and expel more whey. The increase in temperature also speeds up the metabolism of bacteria enclosed within the curd. Lactic acid production increases, the pH declines, and this acidity assists in shrinking the particles to expel more whey.

The optimal cooking temperature for Cheddar cheese is 37°C. Cooking continued for a period ranging from 1 to 1.5 h. The pH of the curd and the whey was checked every 15 min.

2.2.4.8. Draining or Dipping

When the curd pH was 6.0, the whey was removed by draining. This process permanently separates the whey from the curds. The curds were bundled in two layers cheesecloth and squeezed out as much whey as possible.

2.2.4.9. Curd Knitting or Cheddaring

The curds were then spread into the floor of the aluminium container or cheese vat at an angle in the form of 4 to 5 blocks. The temperature of the container was maintained at 38-39°C. The blocks were turned, piled and repiled on top of one another every 15 min for a total of 6 turns. The process of piling and repiling of blocks of warm curds in the cheese vat is known as cheddaring.

During this period, lactic acid increases rapidly to a point where coliform bacteria are killed by the free hydrogen ions. Futhermore, as the curd blocks were repiled their structure flattens, and any holes or eyes originally present lose their identity in the deformed curd. Therefore, the step of cheddaring controls the moisture and transforms the curd into the characteristic texture of the Cheddar cheese. The whey released during cheddaring was periodically removed by using a pasteur pipette. The pH of the curd was checked and after the final turn the pH should be between 5.3-5.4.

2.2.4.10. Milling

After cheddaring, solidified curd was broken up or "milled" and salt was mixed into the curd before it was finally pressed into shape. The curd was cut with a sterilized knife into small cubes of about 1 cm thick. The milling stage also provides for aeration and cooling of the curd. After milling, the curds were weighed and the weight was recorded.

2.2.4.11. Salting

Coarse salt (sodium chloride) was added to the curds to suppress the growth of unwanted bacteria, to control the growth of wanted micro-organisms and thus the rate of ripening, to assist the physico-chemical changes in the curd, and to give flavour to the cheese. The amount of coarse salt added was 2.3 g/100 g of curd.

2.2.4.12. Pressing

In the pressing stage, the curd was confined in a wooden cylinder under external pressure from the top. The main aim of cheese pressing is to form the loose curd particles into a shape which is compact enough to be handled, and to expel any free whey, and complete the curd knitting.

Pressing the curd should be gradual at the begining because high pressure at first compresses the surface layer of the cheese and can lock moisture into pockets in the body of the cheese.. The pressure applied to the cheese should be per unit area of the cheese and not per cheese which may vary in size. The amount of pressure applied to the Cheddar cheese was 56.25 kg/cm^2. The Cheddar cheese was pressed for a period of 24 h.

2.2.4.13. Ripening

At the end of pressing, the cheese was weighed and the weight was recorded. The finished product was vacuum-packaged in Cryovac bags and stored in an incubator set at a temperature between 5-7°C for aging. The chemical and physical changes during the process of ripening of cheese have been described in section 1.5 of the Introduction.

2.2.5. Moisture Content Determination

The moisture content of the Cheddar cheese was determined by using the conventional oven method as described in the Official Methods of analysis published by the Association of Official Analytical Chemists (AOAC, 1980a) with some modification and changes.

A clean and dried round, flat-bottom weighing vial together with the lid was weighed in an analytical balance. It was then dried in an oven at a temperature of 104°C for 2 h. The vial and its lid was taken out from the oven and cooled in a dessicator. When it was cooled, the vial and the lid were weighed in the balance and the weight was recorded. This procedure was repeated several times until the subsequent weighing showed not more than 0.05% loss in weight.

About 2-3 g of the cheese sample was put into the vial and the vial was covered with a lid. It was then dried in the oven with the lid opened. The temperature of the oven was set at 104°C and the sample was dried for 24 h The sample was taken out of the oven and cooled in the dessicator and then reweighed. This procedure was repeated until a constant weight was obtained. The loss in weight in subsequent weighing was expressed as the moisture content of the cheese. A duplicate sample was used for both the protease treated cheese and the control cheese.

2.2.6. Fat Determination

The fat content of the Cheddar cheese was determined by the Babcock method which was described by D.M. Irvine in "Cheddar Cheese Manufacture", a manual published by Ministry of Agriculture and Food, Ontario, Canada. Some modifications and changes in the procedure were made.

A 9 g representative cheese sample was weighed into a Paley bottle using a spatula with the flattened end for the transfer. A 10 mL aliquot of hot distilled water (150°F or 65°C or above) was added. The stopper was inserted and it was shaken well in order to help in breaking up the curd. A 17.5 mL aliquot of concentrated sulphuric acid was added a few mL at a time. The solution was mixed thoroughly after each addition. Final colour of the solution was chocolate brown. It was let to stand undisturbed at room temperature for 4 min. The Paley bottle containing the solution was centrifuged for 5 min at 600 rpm in a Optima II BHG centrifuge. After that, water at a temperature of 150°F (60°C or above) was added up to the base of the reading tube. The contents of the bottle was mixed thoroughly. It was centrifuged again for another 2 min. Hot water was added until the fat column was within the graduated portion of the reading tube. It was centrifuged for another 1 min. The bottle was then placed in a water bath set at a temperature of 54 to 60°C for a period of 5 min. Two or three drops of glymol (or paraffin oil) was added and the percentage of fat was read from the graduated portion of the reading tube. A duplicate sample of each representative sample of the protease treated as well as the control Cheddar cheese were used for the fat determination. The average of the reading of each representative sample was expressed as the fat content of the cheese.

2.2.7. Total Nitrogen Determination

The total nitrogen of the Cheddar cheese was determined by Macro-kjeldahl method as described in the AOAC Manual, 1980b with some modifications and changes.

2.2.7.1. Digestion

Tecator Digestion System DS-6 model (Tecator AB. Box 70, S-26301 HÖGANAS, Sweden) was used. A 0.5-1.0 g of the cheese samples (both the protease treated and the control cheese in duplicate) were weighed into the digestion tubes. An empty digestion tube was used as a blank. For each tube, 2 kjeltabs and 10 mL of concentrated nitrogen-free sulphuric acid was added. The tubes were then placed in a digester which consists of an electrically heated and thermostated alloy block with room for up to 6 digestion tubes. The tubes were then covered with glass exhaust caps. Normally, the digestion temperature selected was in the range of 370-420°C. The tap was turned on for maximum air flow through the Exhaust System. Heat shields were placed on the digester, one at the front of the digestion tubes and the other at the rear of the tubes. The heat shield was used to increase the temperature in the digestion tubes above the blocks. It usually took about 30 min for the digester to reach the working temperature after it had been switched on. The mixture was heated gently until frothing ceased. After about 1 h, it was heated briskly. Once digestion was under way, the acid would reflux high up the tube walls, and any residue would be washed down. If the exhaust airflow was kept down during the later part of the process, this refluxing would be aided. Digestion was completed when the material inside the digestion tube was colourless. The digestion was continued for a short time after the material was colourless. It usually required about 2 h to complete the digestion. The tubes should be rotated at interval during the digestion. The samples should not be allowed to char. It might be necessary to add more sulphuric acid during the digestion.

Once digestion was completed, the tubes were lifted out of the block with a gloved hand complete with their exhaust caps, and allowed to stand in the stand to cool for 10 min. A 75 mL of distilled water was added to the tubes. The tubes were swirled as the water was added in order to avoid the precipitation of the sulphate. They were covered with parafilm until distilled.

2.2.7.2. Distillation

Distillation was carried out in Kjeltec System 1002 Distilling Unit (Tecator AB. Box 70, S-26301 HÖGANAS, Sweden). A 50 mL of 40% sodium hydroxide was added to each digestion tube and 25 mL of boric acid solution was added to each receiver tube. A digestion tube with a digested cheese sample and 50 mL of 40% sodium hydroxide was attached to the distilling unit. The tube was twisted a quarter of a turn to make sure that it was properly sealed to the rubber adapter. A receiver flask with 25 mL boric acid was placed on the platform and the platform was moved to its upper position. The protection door was pulled down. The handle for dispensing alkali was pulled down once. The set amount of alkali would be dispensed. The steam valve was opened to start the distillation. The timer was set to the time previously determined (about 5 min). When the signal sounded, the platform with the receiver flask was moved to its lower positon and the distillation was let to proceed for a few seconds to clean the tip of the glass tube. The steam valve was then closed and the protection door was opened. The digestion tube was removed and placed on its stand. The receiver flask was also removed from the distillation unit. When removing the digestion tube, the teflon tube should be placed in the metal clip. This made it possible to put on a new digestion tube without touching the teflon tube, which after a normal distillation had some drops of hot alkali. A new digestion tube was attached to the distillation unit and a new receiver flask was placed on the platform and the platform was moved to its upper position. The procedure was repeated in the same manner for all the other samples including the blank. Total volume of distillate collected in each flask were recorded.

2.2.7.3. Titration and Calculation of Results

The samples were then titrated by adding standard 0.1 M sulphuric acid until the indicator turned grey or very faint. The volume of the acid used was recorded. The blank used only a small amount of titration acid. This blank value was subtracted from the sample values. The percentage of total nitrogen was calculated from the formula:

$$\%\ \text{Nitrogen} = \frac{(a-b) \times \text{Normality of acid} \times 14.008}{\text{g sample} \times 10}$$

where a = mL of titration acid for the sample

b = the above mentioned blank value

% Nitrogen was converted to % Crude Protein by multiplying with the Kjeldahl factor of 6.38.

2.2.8. Determination of Citrate-HCl Soluble Nitrogen

Citrate-HCl soluble nitrogen in the cheese samples was determined by the method of Vakaleris and Price (1959). They developed a rapid method for estimating the degree of proteolysis in ripening cheese. The method involved measuring the absorption of ultraviolet light by a clear, sodium citrate-hydrochloric acid extract of cheese at pH 4.4 $\pm$ 0.05; these measurements were closely correlated with the per cent of soluble nitrogen in the cheese extract.

Solutions required were:

Sodium Citrate	0.5 M
Concentrated Hydrochloric Acid	11.6 M
Distilled water	

A 12.5 g of the cheese samples (control and protease treated cheese) were weighed. Each sample was transferred into a clean and dried blendor. 50 mL of 0.5 M sodium citrate solution and 100 mL of distilled water were added. The sample was blended for 7 min at maximum setting. At the end of 7 min, the cheese extract was then transferred to a 250 mL volumetric flask. It was left at room temperature until it cooled down to a temperature of about 20°C.. Distilled water was added to bring the volumes to 250 mL in the volumetric flask. The extract was then mixed thoroughly.

The hydrochloric filtrate consists of the following composition:

Sodium citrate cheese solution	100 mL
1.41 M hydrochloric acid	10 mL
Distilled water	15 mL
pH of the filtrate	4.4 $\pm$ 0.05

The filtrate was centrifuged at 10,000 rpm for 10 min in a RC-5 Superspeed Refrigerated Centrifuge (Dupont Instruments Sorvall, Norwalk, Conn.). After that, the filtrate was filtered through a Whatman No.42 filter paper to obtain a clear sodium citrate HCl filtrate which contained the hydrolyzed portion of cheese protein soluble at pH 4.4. The filtrate was referred to as cheese extract, and the nitrogen present in this extract was termed soluble nitrogen.

2.2.8.1. Spectrophotometric Measurements

An aliquot portion, e.g., 25 mL of the cheese extract, was diluted with the same volume of distilled water; this raised the pH from 4.4 $\pm$ 0.05 to 4.5 $\pm$ 0.05. This dilution was made to bring the absorbance in the range of from 0.3 to 1.0 in which the Beckman Du-8 spectrophotometer (Beckman Instruments, Inc. Scientific Instruments Division. Irvine, CA92713) gave accurate and reproducible results. One-centimeter quartz cuvette was used. The absorbance of the filtrates were measured at wavelengths of 270 and 290 mμ.

The moles of tyrosine and tryptophan were calculated by using the equations given below: (Vakaleris and Price 1959)

$$M_{tyr} = (0.95\ A_{270} - 1.31\ A_{290}) \times 10^{-3}$$

$$M_{trp} = (0.307\ A_{290} - 0.020\ A_{270}) \times 10^{-3}$$

where M_{tyr} = Moles of tyrosine per liter of solution containing the mixture of these two amino acids

M_{trp} = Moles of tryptophan per liter of solution containing the mixture of these two amino acids

A_{270} = Absorbance at 270 mμ

A_{290} = Absorbance at 290 mμ

The blank was made up by using the following composition:

0.5 M Sodium Citrate	10 mL
1.41 M Hydrochloric Acid	5 mL
Distilled Water	111 mL

2.2.8.2. Estimation of Soluble Tyrosine and Soluble Tryptophan in Cheese Extract

Tyrosine and tryptophan present in the cheese extract in the form of free amino acids and in peptide linkages were referred to as soluble. Some of the decomposition products of these two amino acids may contribute to the absorbancy and thus, may be included in these two expressions.

The calculated values were then doubled to give concentration of soluble tyrosine and soluble tryptophan in moles per liter of undiluted cheese extract.

Soluble tyrosine and soluble tryptophan can be expressed as mg per 100 g of cheese by multiplying the concentration in millimoles per liter of cheese extract by the factor of 453 for tyrosine and 510.5 for tryptophan (Vakaléris and Price 1959).

2.2.8.3. Determination of Amino Acid Composition

The total amino acid composition of citrate-HCl soluble fraction was determined by mixing 1 mL of the clear filtrate and 1 mL of concentrated HCl. The solution was put in a hydrolysate tube and hydrolyzed under vacuum at 110°C for 24 h. Free amino acid in the citrate-HCl extract was made up of 3 mL of the clear filtrate only.

Both the physiological free and total amino acid of citrate-HCl cheese extracts were performed with a Beckman model 121 MB Amino Acid Analyzer using the methods described in Beckman bulletin 121 M-TB-013. To determine free amino acids, the citrate-HCl extract was mixed with 4 volumes of 20% sulphosalicylic acid to precipitate protein prior to analysis.

The results of the amino acid analysis were tabulated and the graphs of change in concentration of each individual amino acid was plotted as a function of storage time and listed in the appendix section. Physiological total amino acid was referred to as hydrolysate amino acid in the figures in the appendix section.

2.2.9. Sensory Evaluation of Cheese

Both the protease treated as well as the control cheese were evaluated by the ranking test (Larmond, 1982). The cheese employed for sensory evaluation were aged for 3, 6, and 9 months in an incubator maintained at a temperature of 5-7°C. The panelists were all trained to detect the cheddar flavour of the Cheddar cheese. A commercialy "medium" Cheddar cheese (Kraft Company) was also included in these tests. Six trained panelists were provided with numbercoded cheese samples (3x3x1 cm). They were asked to rank the samples for the intensity of cheddaring flavour. The sample which had the sharpest cheddaring flavour was ranked first. The sample which had the second sharpest cheddaring flavour was ranked second and so on. The ranks were converted to scores according to the method of Fisher and Yates (1949). Analysis of variance (ANOVA test) was also carried out. The results of the scores were subjected to statistical analysis to determine whether the samples were significantly different at the 5% and 1% levels (Larmond, 1982). A sample of the taste panel form was attached in the appendix section.

In addition, a preference test for the experimental cheeses at different period of aging was also carried out by the trained panelists. Samples were rated on a 9 point scale where 9 corresponds to "like extremely well" and 1 corresponds to "dislike extremely". They were also asked to comment on the texture and any off-flavour or bitterness of the cheese samples. The results were tabulated.

In order to determine the effect of different concentrations of bacterial protease (T25) on the quality of Cheddar cheese (especially in terms of cheddaring flavour, and texture), Cheddar cheeses with different concentrations (5 mg/L, 10 mg/L and 20 mg/L) were made using the same method and also subjected to the same treatment. They were stored at a temperature of 10°C for ripening. Sensory evaluations were carried out at 3, 6, 9 and 12 months using the above method. The results were tabulated.

2.2.10. Determination of pH of Cheese Samples

Cheese samples (3 g approximately) were grated into an open sterile petri plate and then transferred into a tube. The electrode of a pH meter was pressed into the tube until a layer of cheese completely covered the electrode. When a stable pH reading was observed, it was recorded.

RESULTS

2.3. Chemical Composition of the Cheese Samples

2.3.1. Moisture Content

Moisture content of the cheese samples was determined by the method described in chapter 2, section 2.2.5. The results was tabulated in the following table:

Table 2-1: Moisture Content of the Cheese Samples

Cheese Sample	Moisture Content (%)[1]	
	Batch 1	Batch 2
Protease	37.47	35.90
Control	43.80	39.82
Commercial[2]	34.0	31.30

[1]Data are the mean values for duplicate determination for one lot of cheese.
[2] Kraft "medium" Cheddar cheese. The commercial cheese sample was purchased at the time of sensory evaluation and did not reflect the aging.

2.3.2. Fat Content

Fat content of the cheese samples was determined by the method described in chapter 2, section 2.26. The results were given in the following table:

Table 2-2: Fat Content of Cheese Samples

Cheese Sample	Fat Content (%)[1]	
	Batch 1	Batch 2
Protease	27.0±1.00	29.5±0.60
Control	30.0±0.50	33.0±1.00
Commercial[2]	33.2	33.2

[1]Data are the average of 3 analysis for one lot of cheese.
[2] Kraft "medium" Cheddar cheese. The commercial cheese sample was purchased at the time of sensory evaluation and did not reflect the aging.

2.3.3. Total Nitrogen and Protein Content of Cheese Samples

Total Nitrogen and Protein Content of Cheese Samples were determined by the method described in chapter 2, 2.2.7. The results were given in the following table:

Table 2-3: Total Nitrogen and Protein Content of Cheese Samples

Cheese Sample	Total Nitrogen (%)		Crude Protein Content (%)[1]	
	Batch 1	Batch 2	Batch 1	Batch 2
Protease	3.61	3.40	23.0	21.7
Control	4.28	3.63	27.3	23.2
Commercial[2]	4.15	4.15	26.5	26.5

[1]Protein was calculated by multiplying Kjeldahl N by a factor of 6.38.
Data are mean values of duplicate determination for one lot of cheese.
[2] Kraft "medium" Cheddar cheese. The commercial cheese sample was purchased at the time of sensory evaluation and did not reflect the aging.

2.3.4. pH values during aging of Cheddar cheese samples

pH of Cheese samples during aging was determined by the method described in Chapter 2, section 2.2.10. and the results were given in the table below:

Table 2-4: Average pH values during ripening of Cheddar cheese

	Time of ripening (months)			
Cheese Samples	0	3	6	9
Protease (T25)	4.95a	5.20b	5.35c	5.38c
Control	4.90a	5.32b	5.52d	5.60d

Data are mean values for triplicate determination for one lot of cheese.
Values bearing different letters differ significantly at $P<0.01$ level.
Comparisons are made by columns and rows.

2.3.5. Sensory Evaluation of Cheddar Cheese Samples

Sensory evaluation of Cheddar Cheese Samples were conducted using the method described in Chapter 2, section 2.2.9. The results of the sensory evaluation scores at the aging period of 3, 6 and 9 months old Cheddar Cheese samples were given in the table below:

Table 2-5: Sensory Evaluation Score for Cheddar Cheese Samples (Batch 1)

Cheese Samples	Mean rank score* Time (months)		
	3 months	6 months	9 months
Protease (T25)	+0.65a	+0.75c	+0.83e
Control	-0.05a	-0.14d	-0.17d
Commercial[2]	+0.30b	+0.35bc	+0.40bc

*Numbers followed by the same letter are not significantly different at $P<0.01$ level. Comparisons are made by columns and rows. n=6. 1=sharpest cheddaring flavour.

[2] Kraft "medium" Cheddar cheese. The commercial cheese sample was purchased at the time of sensory evaluation and did not reflect the aging.

Table 2-6: Sensory Evaluation Score for Cheddar Cheese Samples (Batch 2)

Cheese Samples	Mean rank score* Time (months)		
	3 months	6 months	9 months
Protease (T25)	+0.58a	+0.77c	+0.85c
Control	-0.08a	-0.18d	-0.20d
Commercial[2]	+0.29b	+0.34bc	+0.38bc

*Numbers followed by the same letter are not significantly different at $P<0.01$ level. Comparisons are made by columns and rows. n=6. 1=sharpest cheddaring flavour.

[2] Kraft "medium" Cheddar cheese. The commercial cheese sample was purchased at the time of sensory evaluation and did not reflect the aging.

Table 2-7: Preference test for Cheddar Cheese Samples

Cheese Samples	Mean score* Time (months)		
	3 months	6 months	9 months
Protease	6.67a	7.46d	8.15h
Control	5.32b	5.01e	4.70i
Comercial[1]	8.00c	8.15c	8.54c

[1] Kraft "medium" Cheddar cheese. The commercial cheese sample was purchased at the time of sensory evaluation and did not reflect the aging.
*Values followed by the same letter are not significantly different at $P<0.01$ level. Comparisons are made by columns and rows. n=6. 9=like extremely and 1= dislike extremely.

2.3.6. Yield of Cheddar Cheese Samples

The yield of the Cheddar Cheese Samples were calculated in terms of wet weight and dry weight of the curd. The dry weight was determined at the end of the pressing period. Wet weight was determined before pressing. The results were recorded on the Table 2-9.

Table 2-8: Sensory evalution of Cheddar cheese made with porcine pepsin and different concentrations of bacterial protease (T25)

Cheddar flavour Intensity rank	Sample	Process	Aging time (Months)
1a	Kraft(medium)	conventional	6-12
2a	Porcine pepsin	conventional	3
3b	PP[1]+T25(10 mg/L)	conventional	3
4c	PP+T25 (5 mg/L)	conventional	3
5c	PP+T25 (20 mg/L)	conventional	3
1a	PP+T25 (10 mg/L)	conventional	6
2a	PP+T25 (20 mg/L)	conventional	6
3b	PP+T25 (5 mg/L)	conventional	6
4b	Kraft (medium)	conventional	6-12
5c	Porcine pepsin	conventional	6
1a	PP+T25 (20 mg/L)	conventional	9
2a	PP+T25 (10 mg/L)	conventional	9
3b	PP+T25 (5 mg/L)	conventional	9
4b	Kraft (medium)	conventional	6-12
5c	Porcine pepsin	conventional	9
1a	PP+T25 (20 mg/L)	conventional	12
2a	PP+T25 (10 mg/L)	conventional	12
3b	PP+T25 (5 mg/L)	conventional	12
4b	Kraft (medium)	conventional	12
5c	Porcine pepsin	conventional	12

[1]PP=Porcine pepsin.
Numbers followed by the same letter are not significantly different at $P<0.05$ level. n=6. Comparisons are made by rows only.
Kraft "medium" Cheddar cheese. The commercial cheese sample was purchased at the time of sensory evaluation and did not reflect the aging.

Table 2-9: Yield[1] of Cheddar Cheese Samples

Cheese Sample	Wet Weight (g) Batch 1	Dry Weight (g) Batch 1	Wet Weight (g) Batch 2	Dry Weight (g) Batch 2
Protease (T25)	956.1	856.1	730.0	656.7
Control[2]	1000.3	985.9	886.7	845.5

[1]Yield of cheese (dry weight) from 8 liters milk.
[2]No T25.
Wet weight refers to weight before pressing and the dry weight is the weight after pressing. Yield refers to the dry weight.

2.3.7. Amino Acid Composition

Both the hydrolysate and free amino acid of citrate-HCl cheese extracts during ripening were determined by the method described in Chapter 2, section 2.2.8.3. The results of the amino acid analysis were given in the tables listed in the appendix.

DISCUSSION

The presence of free amino acids in the curd of a ripening cheese play a very important role in the contribution to the general background flavour of a specific type of cheese (Mulder 1952, Scott 1981, and Virtanen *et al.*, 1948.). Free amino acid changes in great amounts and various proportions during the ripening process. Many of them increase greatly during the period of ripening; some occur in only small amounts and some are decomposed very rapidly by specific enzymes. Numerous publications consolidate these findings on the dynamic aspects of the ripening process (Ali; 1960, Ali and Mulder, 1961).

Free amino acids have been thought to indicate the extent of ripening in cheese. One of the functions of the free amino acids is to form a "pool" from which other components of flavour or aroma are formed (Scott, 1981). Many of these are amines formed by decarboxylases and have included putrescine, cadaverine, histamine, taurine, asparagine, glutamine and tryptamine (Scott, 1981).

Depending on the individual taster, amino acids have a distinct first taste, although an aftertaste may differ slightly. The first taste of the following amino acids appears bitter - methionine, histidine, lysine, tryptophan, leucine, isoleucine, arginine, phenylalanine. Tyramine which is not an amino acid but a derivative from tyrosine also contribute to the bitter taste.

The following amino acids appear to be sweet - serine, glycine, alanine, hydroxyproline, proline, aminobutyric acid, valine, threonine. The following are "broth-like" in taste - aspartic acid, glutamic acid, while the following have little or no taste - asparagine, glutamine, tyrosine. A rubbery taste is given by cystine (Scott, 1981).

Peptides from the degradation of proteins exhibit flavours according to those amino acids which are terminal in the peptide chain (Scott, 1981).

The keto acids produce compounds leading to distinctive flavours or aromas and are formed from lactose, fatty acids, proteins or amino acids and include various aromatic and aliphatic ketones (Scott, 1981).

The ketones are similar to the aldehydes in regard to their reactions and both groups have intense flavour or aromatic characteristic (Scott, 1981).

Law *et al.* (1976a) considered amino acids to be the intermediate products in the production of certain aroma compounds in cheese. Therefore, the measurement of the levels of free amino acids is useful in the investigation on flavour intensity of Cheddar cheese.

"There is a trend in the relation between the age of cheese and the soluble tyrosine in the cheese extract." (Vakaleris and Price, 1959). Previous studies have shown that the flavour intensity of Cheddar cheeses positively correlates with free amino acid content and the content of tyrosine and soluble peptides containing tyrosine (Vakaleris and Price, 1959). The results of our studies indicated a fluctuation of the concentration in hydrolysate amino acids as well as free amino acids during ripening period of Cheddar cheese as shown in Fig.A-1 and Fig.A-2.

All the figures referred to in this discussion are in the Appendix section. The overall change in free amino acids in protease treated Cheddar cheese was slightly higher than that of the control (as shown in Fig.A-2). Individual free and total amino acids showed fluctuation in great amounts and also in various proportions during the ripening process (Fig.A-3 - Fig.A-46). Many of them increased greatly during ripening; some occurred in only small amounts and some were decomposed very rapidly. Our results indicated that the free amino acids in citrate-HCl extract that increased during the ripening process are phenylalanine, glycine, isoleucine, leucine, threonine, lysine and glutamic acid. The changes in the free amino acids during the ripening period may be due to enzymatic degradation of peptides by various microorganisms and also from amino acid interconversion, excretion and degradation (Polo *et al.*, 1985).

Concentration of soluble tyrosine (mg/100 g of cheese) reached its maximum peak in 6 months for the protease treated cheese (Fig.A-47). Sensory evaluation conducted at 6 months ripening time showed that the cheddaring flavour of the protease treated cheese was significantly higher than that of the control cheese at both 5% and 1% level (Table 2-5). At that period, some trained panelists even indicated that the protease treated cheese had the strongest cheddaring flavour among all the samples. The strong Cheddar flavour may be in part derived from sulfur containing amino acids (Singh and Kristoffersen, 1969; Manning, 1978) or fatty acids (Schormuller, 1968). The significance of sulphydryl concentration in flavour formation and stability has also been pointed out by Kristoffersen (1967). Sulphydryl groups are involved directly in the flavour and also provide the source for hydrogen sulphide. In addition, sulphydryl groups stimulate proteolysis and fatty acid production, thus contributing greatly to the overall flavour control. However, ultimately the development of characteristic cheese flavour appears to be determined by the ability of protein-based sulphur groups to accept hydrogen resulting from oxidative ripening processes (Shankaranarayana *et al.*, 1982).

Bitterness and off-flavour were not detected in all the protease treated cheese throughout the ripening period. All the trained panelists rated all the samples are of good texture during aging. Majority of the trained panelist also rated both the protease treated and the commercial cheese are of good quality in terms of cheddaring flavour, texture and also higher preference score than that of the control cheese.

From the results of the sensory evaluation of effect of different concentrations of bacterial protease (T25) on the cheddaring flavour of Cheddar cheese, our results seems to show that higher concentrations (10 mg/L and 20 mg/L) of bacterial protease (T25) gives rise to higher score of cheddaring flavour during aging of Cheddar cheese. The results also indicates that Cheddar cheese made with bacterial protease (T25) and porcine pepsin contribute to better cheddaring flavour (starting from 6 months of aging) than the cheese made from porcine pepsin and commercial Kraft cheese. According to the comments of the trained panelists, no off-flavour was detected in the protease treated cheese throughout the ripening period and the texture of all the cheeses remained good quality.

The presence of pyschrotrophs or their proteases can result in the reduction of the yield of cheese besides causing other quality defects. Hicks *et. al.*, found that psychrotrophs added to milk used for manufacturing of cheese reduced cheese yield. This loss was attributed to lipolytic and proteolytic activity of the psychrotrophs. In the present study, total fat, protein and nitrogen showed lower values than those in a control cheese without the addition of the bacterial protease (T25). Since protease T25 used in the present investigation was an impure solution, it may have carried lipase as well. Other workers have reported that the breakdown products soluble in whey result in lower cheese yield due to the activity of psychrotrophs (Cousin and Marth, 1977; Hicks *et. al.*, 1982; Law *et. al.*, 1976b; Nelson and Marshall, 1977, and Yates and Elliot, 1977). The result of our study also showed a slight decrease in yield of the Cheddar cheese in protease treated cheese as indicated in Table 2-19. Total nitrogen in whey from milk inoculated with psychrotrophs has been reported to be substantially higher (Cousin and Marth, 1976, and Cousin and Marth, 1977).

In conclusion, the reduction in total protein, fat and nitrogen in Cheddar cheese containing the bacterial protease is consistent with the findings of other workers (Cousin and Marth, 1977; Hicks *et. al.*, 1982, and Yates and Elliot, 1977). According to the results of sensory evaluation, the ability of the protease treated cheese to achieve the highest score of cheddaring flavour within 6 months of aging indicated that bacterial protease T25 isolated from raw milk is promising as a ripening aid for making Cheddar cheese by the conventional methods.

Chapter 3

The Effect Of Microbial Protease On Residual Casein Fractions In Ripening Cheddar Cheese

INTRODUCTION

Chemical and microbial changes occurring during cheese-ripening have been studied widely (Kosikowski, 1978a; and Marth, 1963). Most of these studies have been concerned with either the measurement of compounds formed or identification of microorganisms occurring during the ripening process. Electrophoretic techniques provide an ideal means for a novel approach to the study of cheese-ripening by detecting important changes in the intact caseins of cheese (Ledford *et al.*, 1966). Gel electrophoretic methods are increasingly used to study the nature and extent of casein degradation in cheese.

In this study, DISC-PAGE was used to study the effect of microbial protease (T25) on the residual casein fractions in ripening Cheddar cheese.

MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Preparation of Ornstein Davis Gels

Solutions for Davis Polyacrylamide gel electrophoresis are:

A. 1 N HCl 48 mL, Tris base 36.6 g, TEMED 0.23 mL. Make up to 100 mL. pH 8.9.

B. 1 N HCl 48 mL, Tris base 5.98 g, TEMED 0.46 mL. Make up to 100 mL. pH 6.7.

C. Acrylamide 28 g, bis acrylamide 0.735 g. Make up to 100 mL.

D. Acrylamide 10 g, bis acrylamide 2.5 g. Make up to 100 mL.

E. Riboflavin 4 mg, add distilled water to make up to 100mL.

F. Sucrose 40 g, make up to 100 mL.

G. Ammonium Persulphate 0.14 g, make up to 100 mL.

Running buffer was made up of the following compositon:

Tris base	0.6 g
Glycine	2.88 g

Make up to 1 L with distilled water. Final pH was 8.3

Sample buffer contained the following composition:

Glycerol	10 mL
2-mercaptoethanol	5 mL
Urea	36.036 g

Make up to 100 mL with distilled water.

Lower gel was made up of the following compostion:

Solution A	5 mL
Solution C	10 mL
Distilled water	5 mL
Solution G	20 mL

Stacking gel was made up of the following composition:

Solution B	1 mL
Solution D	2 mL
Solution E	1 mL
Solution F	4 mL

Staining solution was made up of the following composition:

Coomassie brilliant blue	1.25 g
Methanol	227 mL
Glacial acetic acid	46 mL

Make up to 500 mL with distilled water and filter.

Destaining solution was made up of the following composition:

Methanol	50 mL
Glacial acetic acid	75 mL

Make up to 1 L with distilled water

3.2. METHOD

Twelve gel tubes (0.5 x 13 cm) were cleaned by rinsing them in Photoflo and dried in the oven for 2 h. At the end of the drying period, the gel tubes were taken out of the oven and one end of the tube was covered with parafilm and placed in a level gel rack. They were filled with lower gel solution up to the 8.5 cm mark with a pasteur pipette. Distilled water (200 μL) was laid over the gel carefully. They were left at room temperature undisturbed for 30 min to polymerize or solidify. When the gels were solidified, the distilled water was removed with a pasteur pipette, and 10 drops of stacking solution was added to each gel. Distilled water (200μL) were carefully laid on top of each gel. The gels were then left to polymerize at room temperature. When the gels were polymerized, the water on top of the gels was removed by using a Pasteur pipette. The parafilm was removed from the end of the gel and the gels were placed in a Bio-Rad Model 155 gel electrophoresis cell. The adapters were placed in the chamber of the electrophoresis cell. The upper and lower sections of the chamber were filled with running buffer.

A 0.2 g of each cheese sample (both protease and control cheese) were added to 5 mL of 0.2% sodium citrate containing 0.2% of 2-mercaptoethanol. The mixture was left undisturbed at room temperature for 1 hour. At the end of 1 hour, it was vortexed and 3.6 g of urea were added. The mixture was heated in a water bath set a temperature of 50°C. When all had dissolved, distilled water was added to make the volume up to 10 mL.

Standards were used for comparison. They were soluble whole casein, α-casein, β-casein, γ-casein and κ-casein (Sigma Company). Standards proteins were also used and cheese sample were used in duplicate.

Protein samples (50μL) were added to 50 μL of sample buffer containing 6 M urea. Three drops of glycerol followed by 10 μL of 0.1% bromophenol blue tracking dye were added to each sample. Each sample was pipetted into the gel tubes inside the chamber. Running buffer was carefully overlaid on top of the gel tubes. Initially, a current of 1 mA was applied to each gel tube using a Buchler 3-1500 Constant Power Supply. The current was increased to 2 mA per gel tube when the samples entered the portion of the running gel. The electrophoresis was judged to be complete when the bromophenol blue dye was 1 cm from the bottom of the tube. The gels were removed immediately from the glass tubes using a syringe filled with distilled water fitted with a 21 gauge needle and were fixed in 14 mL of 12% trichloroacetic acid solution for half an hour. The TCA solution were poured away and the gels were stained in Coomassie Brilliant Blue solution overnight. Then the gels were destained in destaining solution in a diffusion destainer containing activated charcoal (Bio-Rad). Quantitation was attempted by desitometric scanning of the gels at A_{590} using DU-8 Spectrophotometer.

DISCUSSION

In 1977, Visser and deGroot-Mostert studied the effect of proteolysis in Gouda cheese using electrophoretic techniques. They found that in normal aseptic Gouda cheese, α_{s1}-casein was degraded rapidly and the degradation was completed after one month of ripening. β-Casein was more resistant to proteolysis. After 6 months of ripening, about 60% of β-casein was still intact. Law (1981) studied the differences in casein breakdown patterns between control and enzyme-treated cheeses using PAGE gels scanned at A_{590}. He found out that cheese treated with larger amount of proteinases contained progressively less β- and α_{s1}-casein.

According to the results of our present study of the effect of bacterial protease (T25) on the casein fractions in ripening Cheddar cheese, as can be seen in the electrophoretic densitogrammes, α_{s1}-casein fraction was degraded very rapidly in 4 months old protease treated Cheddar cheese. Only a small peak was observed in the electrophoretic densitogrammes when compared to that of the standard α-casein. α_{s1}-casein fraction was degraded completely in 9 months old Cheddar cheese made by bacterial protease (T25), β-casein appeared to remain undigested during this time as shown in Fig.3-1. For a 4 months old protease treated Cheddar cheese, intensity of the α_{s1}-casein fraction appeared to be more significant than the 9 months old cheese. This results indicated that α_{s1}-casein fraction is degraded during the process of ripening in the protease treated Cheddar cheese. Bacterial protease (T25) may help in the degradation of α_{s1}-casein fraction during the aging of Cheddar cheese. For the control cheese, the effect of degradation of α_{s1}-casein fraction was not significant. According to Mulvihill and Fox (1979) degradation of α_{s1}-casein is desirable for the development of aged Cheddar flavour, whereas, degradation of β-casein may be undesirable and may result in the development of bitter flavour. The ability of the protease treated Cheddar cheese to develop its Cheddar flavour may be due to the degradation of α_{s1}-casein fraction. The action of T25 may be due to proteolytic action rather than to contaminating lipase or nutrients. It could also be due to indirect stimulation of bacterial growth and consequent bacterial/ enzyme action.

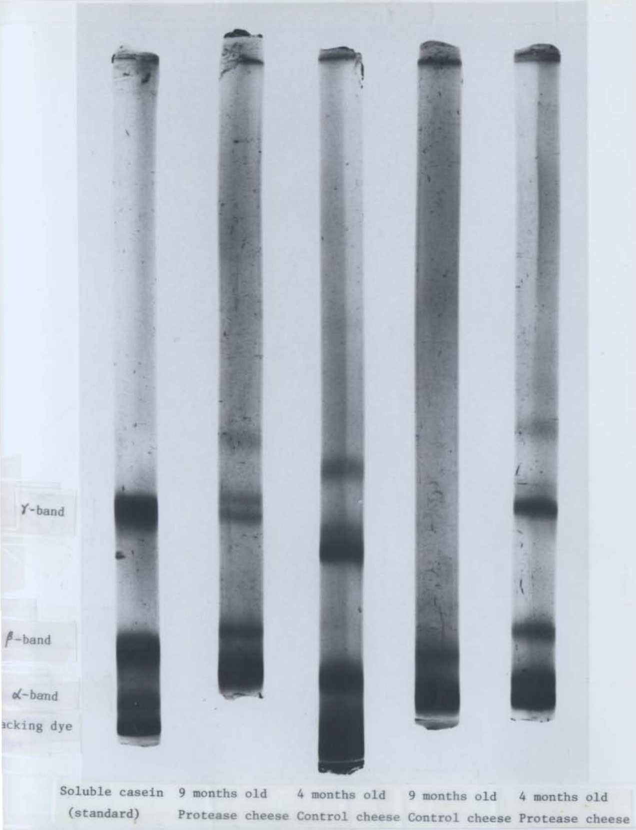
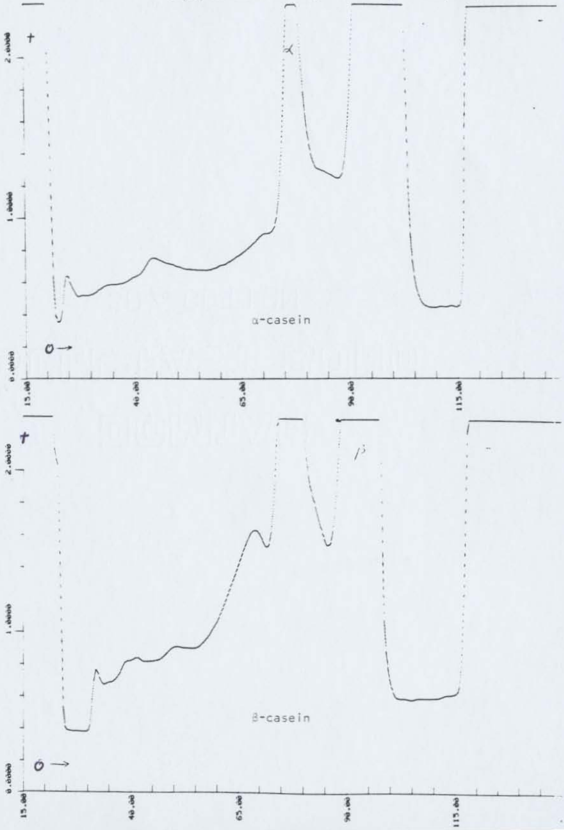
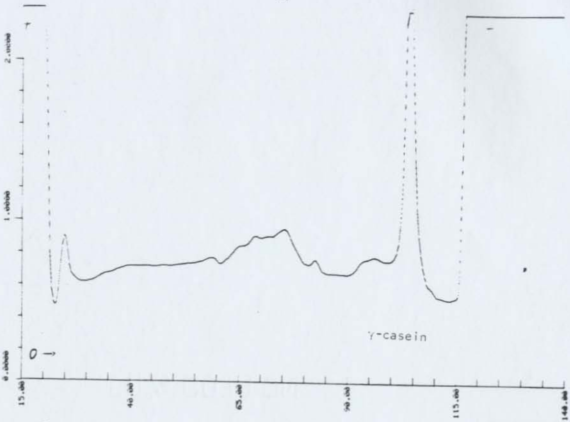


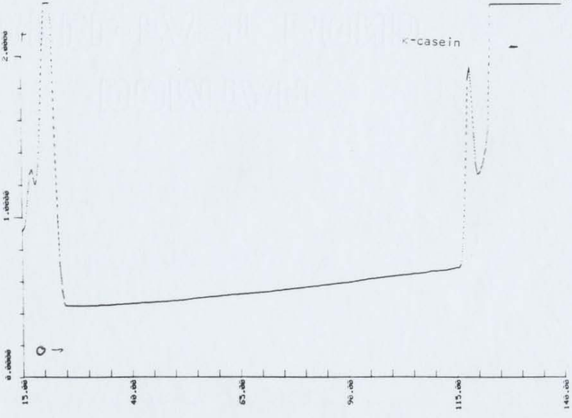
Figure 3-1: Degradation of α_{s1}-casein fraction by bacterial protease (T25) in ripening Cheddar cheese using DISC-PAGE

Figure 3-2: Electrophoretic densitogrammes showing the degradation of α-(s1)-casein fraction by bacterial protease (T25)

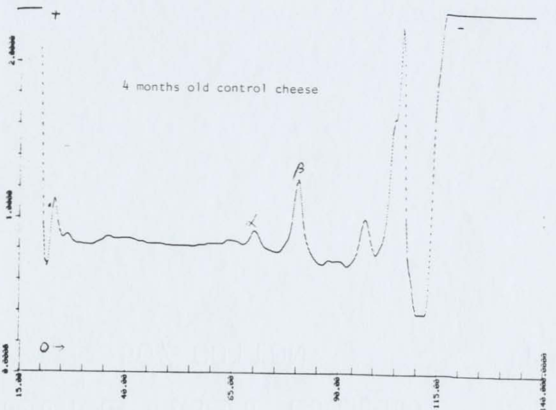




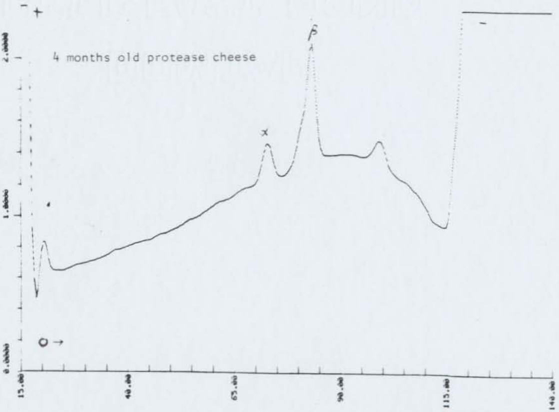
2.0000
1.0000
0.0000
γ-casein
15.00
40.00
65.00
90.00
115.00
140.00



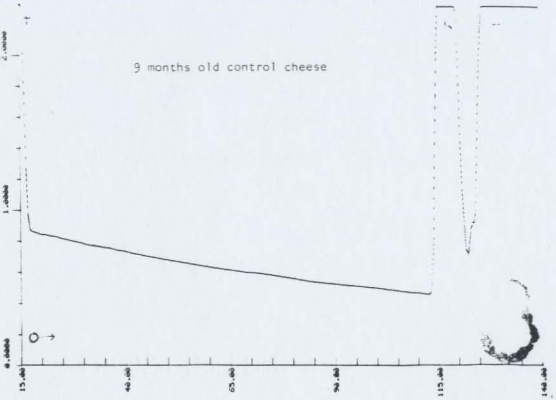
2.0000
1.0000
0.0000
κ-casein
15.00
40.00
65.00
90.00
115.00
140.00

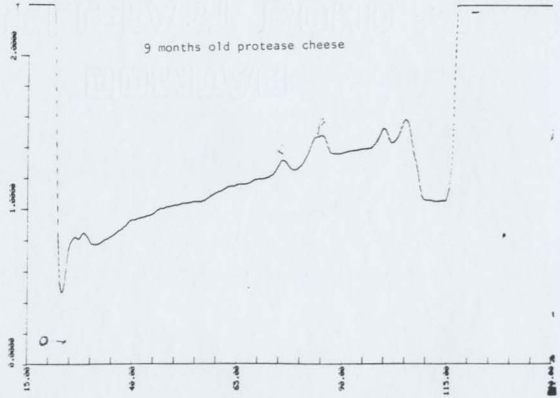


4 months old control cheese
β
κ
O →
2.0000
1.0000
0.0000
15.00
40.00
65.00
90.00
115.00
140.00



4 months old protease cheese
β
κ
O →
2.0000
1.0000
0.0000
15.00
40.00
65.00
90.00
115.00
140.00


9 months old control cheese
2.0000
1.0000
0.0000
15.00
40.00
65.00
90.00
115.00
140.00


9 months old protease cheese
2.0000
1.0000
0.0000
15.00
40.00
65.00
90.00
115.00

In order to determine whether T25 degrades α-casein but not β-casein when incubated with whole casein or milk substrate, an *in vitro* experiment had been carried out previously in our laboratory to determine the substrate specificity of proteases by using the methodof Patel *et al.*, 1983a. The results were listed in the table below.

Table 3-1: Substrate specificity of the proteases from psychrotrophic Pseudomonas.[1]

Substrate	% Relative activity					
	T6	T10	T16	T18	T20	T25
Casein	100	100	100	100	100	100
α-casein	nd[2]	104	143	89	120	125
β-casein	93	84	80	59	77	96
γ-casein	57	59	69	54	41	51
κ-casein	98	nd	nd	90	14	nd
BSA[3]	32	50	0	12	6	0
Ovalbumin	7	0	0	0	0	0
Hb[4]	18	0	9	0	36	9

[1]The protease activity obtained in the presence of soluble casein as a substrate was expressed as 100%. Activity detected in the presence of other substrates is relative to that obtained with soluble casein.
[2]nd=not determined.
[3]BSA=Bovine Serum Albumin
[4]Hb=Hemoglobin.

According to the table above, extracellular proteases degraded α-casein much more than that of the β-casein. α-casein was also a better substrate than other proteins.

Chapter 4

The Effect Of Heat-Stable Protease T25 On The Microbial Counts In Aging Cheddar Cheese

INTRODUCTION

The ripening of cheese is brought about through the agency of enzyme systems produced by bacteria which have grown or are growing in the curd. The bacterial population of cheese curd is continually changing both in numbers and in species. On the first day of a cheesemaking process the number in the starting material ranged from 1 to 2 billion per gram (Kosikowski, 1985). Thereafter the population declines because of insufficient oxygen, high acidity and the presence of inhibitory compounds that are produced as the cheese ripens. Fortunately, the ripening organisms are safe and perhaps beneficial. It is largely the action of their cellular enzymes on lactose, fat and protein that creates the ripened-cheese flavour.

In this study, we looked at the effect of microbial protease on the bacterial counts in aging Cheddar cheese because heat-stable protease (T25) was used to accelerate the ripening process of Cheddar cheese. In addition, we also investigated the survival of starter culture in mineral salt medium with different concentrations of heat-stable microbial protease.

MATERIALS AND METHODS

4.1. MATERIALS

Samples of raw milk were purchased from a local milk plant (Kelsey J & Sons Ltd). [illegible] of the bacteriological media and reagents were purchased from Difco Laboratories (Detroit, MT); all other chemicals were of analytical grade and were purchased either from Sigma Chemical Co. (St. Louis, Mo) or from British Drug House Ltd.

The chemicals used for total bacterial counts are:

1. 2 % Sodium Citrate
2. Yeast Glucose Agar
3. Distilled Water

Yeast Glucose Agar was prepared with the following composition:

Bacto Yeast Extract	3.0 g
Bacto Tryptone	5.0 g
Bacto Dextrose	1.0 g
Bacto Agar	15.0 g

Distilled water was added to make up a total volume of 1 L. The solution was brought to boiling and then autoclaved at 15 lbs. per square inch for 15 min. It was taken out of the autoclave and cooled to 40-50°C before pouring into the petri plates.

4.2. METHODS

4.2.1. Source of Microbial Protease

A type of heat-stable microbial protease was used in the making of Cheddar cheese. ***Pseudomonas fluorescens*** (T25) isolated from raw milk was used as a source of this enzyme.

4.2.2. Enzyme Preparation and Protease Assay

Please refer to chapter 2 section 2.2.2 and 2.2.3.

4.2.3. Cheddar Cheese Making

Please refer to chapter 2 section 2.2.4.

4.2.4. Determination of Total Bacterial Counts in Cheddar Cheese

Cheese samples (5.5 g, both the protease and the control) were weighed into a sterile blender. A 50 mL aliquot of 2% sodium citrate was added. The cheese samples were then blended for 2 to 3 min in the sterile blender until a homogenized solution was obtained. They were then transferred into a sterile Erlenmeyer flask aseptically. One mL of the sample was aseptically pipetted from the flask into a sterile test tube containing 9 mL of 2% sodium citrate. Serial dilutions were made until a final dilution of 10^{-6}. One mL of the diluted sample (10^{-4} to 10^{-6}) was pipetted in duplicate into empty petri plates aseptically. Yeast glucose agar was poured into the petri plates. The contents were swirled gently. The plates were left at room temperature until they solidified. They were then incubated at 37°C for 48 h before colony forming units (CFU) per plate were counted by means of a colony counter.

4.2.5. The Survival of Starter Culture in Mineral Salt Medium With Different Concentrations of Protease

Preparation of solutions for MSM medium

III A	NH_4Cl	1.0 g
	K_2HPO_4	4.355 g
	$NaH_2PO_4.H_2O$	3.450 g

The final solution was made up to a volume of 1 L with distilled water. The pH of the final solution was adjusted to 6.8.

III B	$MgSO_4.7H_2O$	48.0 g/L = 0.195 M
VIII B	III D + 3.6 g/L $CaCl_2.2H_2O$	
III D	$(NH_4)_6MO_7O_{24}$	0.02 g
	$FeSO_4.7H_2O$	0.02 g
	$MnCl_2.H_2O$	0.10 g
	$CuCl_2.H_2O$	0.10 g
	Concentrated HCl	0.5 mL

The final solution was made up to a volume of 1 L with distilled water. Solutions III A, III B and VIII B were autoclaved separately.

The complete Mineral Salt Medium (MSM) has the following concentrations:

100 mL of III A

1.0 mL of III B

1.0 mL of VIII B

10 g/L of succinate (autoclaved)

Procedure

Three 250 mL Erlenmeyer flasks were autoclaved. Inoculum (0.5 mL) was grown in a sterilized flask containing 45 mL MSM and 5 mL succinate. They were incubated at 25°C in a shaker overnight. At the end of the incubation period, the required amount of protease (T25) were inoculated aseptically and the absorbance was measured at a wavelength of 600 nm for every 3 h interval until a decline in the growth curve was observed. This experiment was repeated twice and identical results were obtained.

Flask A (Control)

45 mL MSM + 5 mL succinate + 0.5 mL inoculum

Flask B

45 mL MSM + 5 mL succinate + 0.5 mL inoculum + 1.0 mL protease

Flask C

45 mL MSM + 5 mL succinate + 0.5 mL inoculum + 2.0 mL protease

RESULTS

4.2.6. Results for Total Bacterial Counts in Cheddar Cheese made from Porcine Pepsin and Microbial Protease (T25)

The results for Total Bacterial Counts in Cheddar Cheese made from Porcine Pepsin and Microbial Protease (T25) are tabulated and listed in the appendix section.

4.2.7. Results of the growth of starter culture in Mineral Salt Medium with different concentrations of protease

The results of the growth of starter culture in Mineral Salt Medium with different concentrations of protease were listed in the appendix section. Graphs of absorbancy at 600 nm versus time in hours were plotted for different concentrations of the T25 microbial protease and the control.

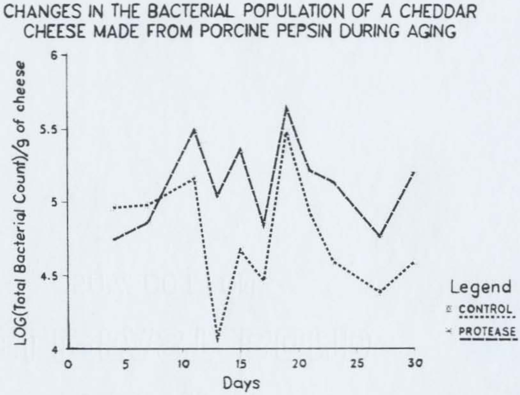
DISCUSSION

4.3. Bacterial counts in aging Cheddar Cheese

Fluctuation in the total bacterial counts was observed during the first 30 days when porcine pepsin was used as a renneting agent in the manufacture of Cheddar Cheese. The total bacterial count reached its highest numbers in about 20 days. After that there was a gradual drop in total bacterial count until day 26-27 (Fig.4-1). The results indicated that the presence of bacterial protease (T25) did not seem to influence the pattern of the bacterial growth when compared to the control cheese without the protease. However, the overall total bacterial counts in the cheese with added protease were slightly higher than that of the control. This may be due to the additional nutrient factors present in the crude extracts of isolate T25. There is also a possibility that the action of protease may have released products easily metabolized by the growing cells in the cheese. Since the cheese samples were vacuum packaged and stored at 5-7°C the differences in bacterial counts in cheese samples with and without the addition of bacterial protease (T25) may reflect the presence of facultative anaerobes which can thrive in a psychrophilic range of temperatures. The low counts perhaps indicate low density of such bacteria in the cheese. ***Pseudomonas fluorescens*** T25 besides secreting an extracellular protease also secretes lipase(s) as reported earlier by [illegible]el *et. al.*, (1983a). Since protease T25 used in the present investigation was an impure solution it may have carried the lipase as well. This lipase may have influenced the bacterial numbers in the cheese sample and caused the differences in the bacterial counts in the cheese sample containing bacterial protease T25.

According to the results of the effect of bacterial protease (T25) on the growth of starter culture, bacterial protease (T25) apparently had no influence on the growth of the starter cultures (***S. lactis*** and ***S. cremoris***) when grown in a mineral salt medium. The protease also did not appear to interfere with the bacterial cell surface proteins to cause any hindrance in their normal functions as evident from the growth pattern depicted in this study. It is evident from the result that the added protease stimulated the growth of the starter cultures (Fig.4-2). The increase in the growth of the starter culture may be due to the protease solution which carried additional protein substrates. The breakdown products from the surrounding substrates may also account for the increase in the growth of the starter culture.

Figure 4-1: Effect of bacterial protease (T25) on the total bacterial counts in Cheddar cheese made with porcine pepsin as a renneting agent



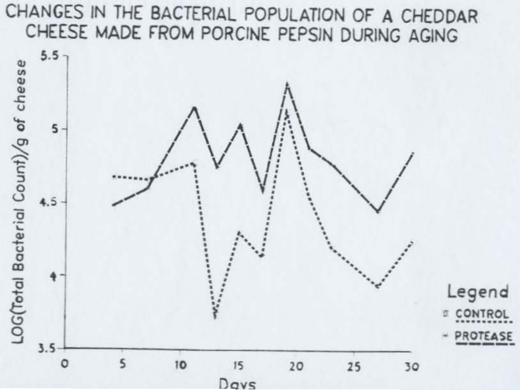
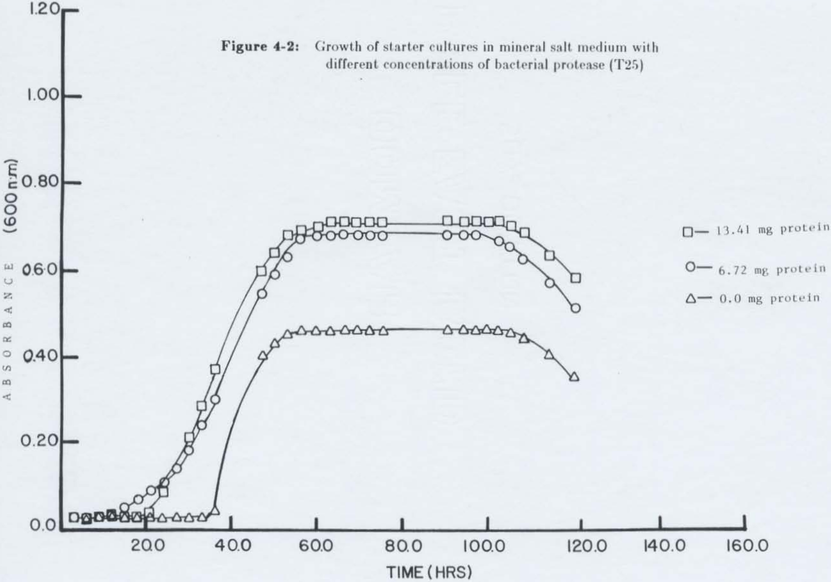


Figure 4-2: Growth of starter cultures in mineral salt medium with different concentrations of bacterial protease (T25)



CONCLUSIONS

The lack of detailed knowledge of cheese flavour compounds, and the mechanisms which generate them during maturation, remains the main obstacle to the rational, scientific design of accelerated cheese ripening systems. However, despite such limitations, a promising development has emerged from our study. According to the results of our present study, several conclusions can be made.

1. The presence of bacterial protease (T25) exerted indirect influence on the growth of the starter cultures by perhaps providing more nutrients in the way of breakdown products from the surrounding substrates.

2. The addition of the bacterial protease (T25) to milk used for Cheddar cheese processing resulted in differences in the growth patterns of bacteria in ripening cheese.

3. The pH changes observed in the control sample during aging were slightly higher than that of the sample containing the bacterial protease.

4. A gradual degradation of α_{s1}-casein fraction was observed in the protease treated cheese during aging.

5. T25 protease gives better activity with α-casein as a substrate compared to other caseins.

6. There was also a trend in the lowering of the percentage of total fat, protein nitrogen, moisture content and yield (dry weight) of the cheese to which bacterial protease (T25) was added.

7. Addition of the protease T25 secreted by ***Pseudomonas fluorescens*** to pasteurized milk prior to the addition of a renneting agent (e.g. porcine pepsin) and preparation of Cheddar cheese by the conventional method results in a product which matures faster than the control cheeses (not containing the added bacterial protease).

Sensory evaluation indicates that the protease treated cheese is also able to achieve a higher intensity of cheddaring flavour ($P<0.01$) and preference score ($P<0.01$) within 6 months of aging compared to the control cheese. Cheddar cheese made with concentration of 10 mg/L and 20 mg/L of bacterial protease (T25) gives higher intensity of cheddaring flavour ($P<0.05$) than that of the lower concentration (5 mg/L). Overall, Cheddar cheeses made from bacterial protease and porcine pepsin are able to achieve higher cheddaring flavour than that of the control cheese during within 6 months of aging. No off-flavour or bitterness was detected in the protease treated cheese throughout the aging period. The texture of the cheeses remained fine during aging.

8. The results of the present study indicates that the protease T25 secreted by ***Pseudomonas fluorescens*** is suitable as a ripening aid for accelerating the ripening process of Cheddar cheese.

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Appendix A

Table A-1: Total Bacterial Counts for the Control Cheese (Without Protease) Batch 1

Time (Days)	TBC[1]/g Cheese	Log_{10}(TBC)/g Cheese
4	91818	4.96
7	96364	4.98
11	143636	5.16
13	11818	4.07
15	46364	4.67
17	28727	4.46
19	301636	5.48
21	83091	4.92
23	39091	4.59
27	24000	4.38
30	39091	4.59

[1]Total Bacterial Counts. Data are mean values for duplicate determination for one lot of cheese.

Table A-2: Total Bacterial Counts for Protease Cheese (Batch 1)

Time (Days)	TBC[1]/g Cheese	Log_{10}(TBC)/g Cheese)
4	54545	4.74
7	72727	4.86
11	306364	5.49
13	109455	5.04
15	227273	5.36
17	69091	4.84
19	437273	5.64
21	160909	5.21
23	133636	5.13
27	57273	4.76
30	157273	5.20

[1]Total Bacterial Counts. Data are mean values for duplicate determination for one lot of cheese.

Table A-3: Total Bacterial Counts for the Control Cheese (Without Protease) Batch 2

Time (Days)	TBC[1]/g Cheese	Log_{10}(TBC)/g Cheese
4	48000	4.68
7	45600	4.66
11	60000	4.78
13	5440	3.74
15	20000	4.30
17	13600	4.13
19	136160	5.13
21	35200	4.55
23	16000	4.20
27	8800	3.94
30	17600	4.25

[1]Total Bacterial Counts. Data are mean values for duplicate determination for one lot of cheese.

Table A-4: Total Bacterial Counts for Protease Cheese (Batch 2)

Time (Days)	TBC[1]/g Cheese	Log_{10}(TBC)/g Cheese)
4	30400	4.48
7	40000	4.60
11	144000	5.16
13	56160	4.75
15	108800	5.04
17	39200	4.59
19	208400	5.32
21	76000	4.88
23	59200	4.77
27	28000	4.45
30	72000	4.86

[1]Total Bacterial Counts. Data are mean values for duplicate determination for one lot of cheese.

Table A-5: Growth of starter culture in MSM

[Protease][1] Time (h)	Flask A 0.0 A_{600}	Flask B 6.72 A_{600}	Flask C 13.44 A_{600}
0	0.020	0.025	0.020
3	0.015	0.028	0.025
6	0.023	0.025	0.025
9	0.025	0.030	0.025
12	0.024	0.035	0.028
15	0.020	0.050	0.030
18	0.020	0.070	0.025
21	0.022	0.090	0.030
24	0.020	0.110	0.085
27	0.025	0.140	0.145
30	0.025	0.185	0.210
33	0.028	0.240	0.285
36	0.040	0.300	0.370
47	0.400	0.545	0.600
50	0.430	0.590	0.640
53	0.455	0.630	0.680
56	0.460	0.670	0.690
60	0.460	0.680	0.700
63	0.460	0.680	0.710
69	0.460	0.680	0.710
72	0.460	0.680	0.710
75	0.460	0.680	0.710
90	0.460	0.680	0.710
94	0.460	0.680	0.710
97	0.460	0.680	0.710
100	0.460	0.680	0.710
102	0.460	0.665	0.710
105	0.455	0.650	0.700
108	0.440	0.625	0.685
114	0.400	0.570	0.650
120	0.350	0.510	0.580

[1][Protease]=Concentration of bacterial protease (T25) in terms of mg of protein.
MSM=Mineral Salt Medium.

Table A-6: Change in Hydrolysate Amino Acids in Citrate-HCl extract During Aging of Cheddar Cheese

Time (Months)	Control (μmol/g)	Protease (μmol/g)
1	47.81	31.99
2	85.45	88.32
3	120.85	97.07
4	75.21	122.31
5	116.36	87.72
6	97.15	61.40
7	160.38	92.16
8	89.95	114.73
9	87.84	153.21
10	89.08	159.0

Data are mean values of duplicate determination for one lot of cheese.

Table A-7: Change in Free Amino Acids in Citrate-HCl extract During Aging of Cheddar Cheese

Time (Months)	Control (μmol/g)	Protease (μmol/g)
1	2.71	3.81
2	6.10	6.95
3	8.92	10.17
4	10.91	14.78
5	13.69	16.24
6	18.24	19.33
7	23.37	23.71
8	28.90	29.43
9	21.28	34.48
10	35.87	37.39

Data are mean values of duplicate determination for one lot of cheese.

Table A-8: Change in Hydrolysate (Alanine-Cystine) in Citrate-HCl extract During Aging of Cheddar Cheese

Time (Month)	Hydrolyzate Amino Acids (umol/g)									
	Alanine C	Alanine P	Arginine C	Arginine P	Aspartic Acid C	Aspartic Acid P	Cysteic Acid C	Cysteic Acid P	Cystine C	Cystine P
1	1.99	1.38	1.37	0.35	5.10	4.51	0.60	0.87	0.12	0.02
2	3.24	3.21	1.81	2.21	10.66	10.55	1.43	0.53	0.12	0.12
3	4.28	3.60	2.44	1.94	14.67	13.38	1.75	2.46	0.22	0.23
4	2.68	4.09	1.33	2.87	10.67	13.24	3.52	2.16	0.07	0.22
5	4.68	3.15	2.63	1.66	13.54	13.23	2.89	5.73	0.00	0.00
6	3.25	1.85	1.56	0.70	12.99	9.25	5.45	5.70	0.13	0.13
7	6.15	4.17	2.92	1.25	17.30	14.90	2.52	4.19	0.32	0.31
8	2.50	3.28	0.65	1.08	13.62	17.28	8.03	7.85	0.24	0.24
9	4.40	5.41	0.84	2.32	8.33	17.34	3.31	2.51	0.00	0.27
10	2.93	6.40	0.60	2.24	12.20	19.90	5.21	5.31	0.22	0.34

C = Control cheese without addition of bacterial protease (T25).

P = Protease cheese with addition of bacterial protease (T25) 9.45 mg/L.

Table A-9: Change in Hydrolysate (Glutamic Acid-Hydroxyproline) in Citrate-HCl extract During Aging of Cheddar Cheese

Time (Month)	Glutamic Acid		Glycine		Histidine		Hydroxylysine		Hydroxyproline	
	Hydrolyzate Amino Acids (umol/g)									
	C	P	C	P	C	P	C	P	C	P
1	11.96	8.40	2.21	1.76	1.18	0.89	0.00	0.00	0.00	0.00
2	21.63	21.68	3.79	4.00	2.14	2.59	0.00	0.00	0.00	0.00
3	32.67	26.39	5.09	4.72	2.83	2.45	0.00	0.00	0.00	0.00
4	19.93	32.50	3.74	5.17	1.84	3.53	0.00	0.00	0.00	0.00
5	28.70	22.57	5.05	4.30	3.16	2.20	0.00	0.00	0.00	0.00
6	24.56	13.25	4.35	2.58	2.26	0.74	0.00	0.00	0.00	0.00
7	40.76	23.29	6.82	4.38	3.77	1.65	0.00	0.00	0.00	0.00
8	19.53	28.04	3.33	4.82	0.76	1.49	0.00	0.00	0.00	0.00
9	22.83	38.30	4.83	5.87	1.89	3.63	0.00	0.00	0.00	0.00
10	16.90	40.21	3.60	6.75	1.06	3.47	0.00	0.00	0.00	0.00

C = Control cheese without the addition of bacterial protease (T25).

P = Protease cheese with the addition of 9.45 mg/L of bacterial protease (T25).

Table A-10: Change in Hydrolysate (Isoleucine-Phenylalanine) in Citrate-HCl extract During Aging of Cheddar Cheese

Time (Month)	Hydrolyzate Amino Acids (umol/g)									
	Isoleucine		Leucine		Lysine		Methionine		Phenylalanine	
	C	P	C	P	C	P	C	P	C	P
1	1.20	0.67	4.25	2.99	3.24	2.08	0.50	0.11	1.41	1.07
2	2.20	2.56	7.83	8.80	5.48	5.77	1.46	1.08	2.67	3.04
3	3.26	2.36	10.85	9.22	7.84	5.65	1.81	1.38	3.64	3.23
4	1.45	3.33	7.60	12.53	5.43	8.26	1.51	2.02	2.57	4.46
5	2.76	1.12	11.36	9.70	9.08	5.91	1.62	2.17	3.77	3.16
6	2.09	0.56	9.19	6.63	7.80	3.72	0.31	1.11	3.10	2.31
7	4.21	1.49	15.45	11.21	12.08	6.24	2.02	1.77	5.38	3.84
8	2.00	2.60	8.68	11.88	6.95	7.15	1.92	2.13	3.13	4.33
9	1.44	3.86	11.65	15.41	6.48	10.89	2.55	1.68	3.68	5.36
10	1.60	4.01	9.19	16.53	7.41	11.03	2.10	3.12	3.22	5.69

C = Control cheese without the addition of bacterial protease (T25).

P = Protease cheese with the addition of bacterial protease (T25) 9.45 mg/L.

Table A-11: Change in Hydrolysate (Proline-Tryptophan) in Citrate-HCl extract During Aging of Cheddar Cheese

Time (Month)	Proline		Serine		Taurine		Threonine		Tryptophan	
	Hydrolyzate Amino Acids (umol/g)									
	C	P	C	P	C	P	C	P	C	P
1	5.00	2.83	2.69	1.28	0.31	0.29	2.04	0.86	0.00	0.00
2	7.74	8.23	3.77	4.11	1.48	1.46	3.03	3.20	0.00	0.00
3	10.27	6.83	6.20	4.17	1.73	1.22	4.33	3.00	0.16	0.13
4	4.58	9.83	2.35	5.94	0.67	1.45	1.85	4.09	0.00	0.00
5	9.06	4.46	6.21	2.73	1.20	0.00	4.53	2.28	0.00	0.00
6	8.85	5.97	3.16	1.32	0.26	1.19	2.69	1.30	0.00	0.00
7	14.15	4.92	8.22	2.36	1.92	2.17	6.12	2.36	0.00	0.00
8	8.80	9.80	1.81	2.68	0.92	0.84	2.00	2.98	0.00	0.00
9	5.74	17.53	2.52	6.38	0.75	2.46	2.10	5.45	0.00	0.00
10	11.33	13.49	0.88	5.10	3.55	2.01	1.66	4.65	0.00	0.00

C = Control cheese without the addition of bacterial protease (T25).

P = Protease cheese with the addition of bacterial protease (T25) 9.45 mg/L.

Table A-12: Change in Hydrolysate (Tyrosine-Valine) in Citrate-HCl extract During Aging of Cheddar Cheese

Hydrolyzate Amino Acids (umol/g)

Time (Month)	Tyrosine		Valine	
	C	P	C	P
1	0.69	0.49	1.89	1.09
2	1.46	1.28	3.31	3.70
3	1.66	1.13	4.92	3.39
4	1.00	1.77	2.29	4.67
5	1.62	0.90	4.53	2.28
6	1.39	1.03	3.66	1.97
7	2.69	1.18	7.04	0.31
8	1.50	1.91	3.54	4.34
9	1.72	1.94	2.79	6.57
10	1.41	1.92	3.95	6.82

C = Control cheese without addition of bacterial protease (T25).

P = Protease cheese with the addition of bacterial protease T25 (9.45 mg/L).

Table A-13: Change in Free Amino Acids (Alanine-Cystine) in Citrate-HCl extract During Aging of Cheddar Cheese

Time (Months)	Free Amino Acid (μ mol/g)									
	Alanine		Arginine		Aspartic Acid		Cysteic Acid		Cystine	
	C	P	C	P	C	P	C	P	C	P
1	0.23	0.35	0.00	0.00	0.16	0.17	0.03	0.03	0.00	0.00
2	0.42	0.45	0.09	0.14	0.37	0.47	0.03	0.54	0.03	0.03
3	0.70	0.83	0.14	0.22	0.01	0.02	0.47	0.48	0.05	0.05
4	0.70	0.96	0.16	0.35	0.17	0.61	0.52	0.66	0.05	0.03
5	0.92	1.06	0.17	0.24	0.35	0.64	1.16	1.20	0.03	0.03
6	1.16	1.22	0.30	0.27	0.57	0.91	0.98	0.70	0.05	0.05
7	1.39	1.41	0.20	0.18	0.74	0.98	0.91	0.93	0.05	0.04
8	1.69	1.65	0.21	0.12	0.85	1.13	1.72	1.87	0.06	0.07
9	1.16	1.86	0.22	0.08	0.60	1.41	1.16	1.84	0.00	0.00
10	2.16	1.93	0.14	0.14	1.15	1.59	2.10	2.11	0.00	0.00

C = Control cheese without addition of bacterial protease (T25).

P = Protease cheese with the addition of bacterial protease T25 (9.45 mg/L).

Table A-14: Change in Free Amino Acids (Glutamic Acid-Hydroxyproline) in Citrate-HCl extract During Aging of Cheddar Cheese

Time (Months)	Free Amino Acid (μ mol/g)									
	Glutamic Acid		Glycine		Histidine		Hydroxylysine		Hydroxyproline	
	C	P	C	P	C	P	C	P	C	P
1	0.67	0.80	0.02	0.08	0.06	0.08	0.00	0.00	0.00	0.00
2	0.99	1.14	0.14	0.12	0.10	0.10	0.00	0.00	0.00	0.00
3	1.89	2.19	0.22	0.26	0.19	0.13	0.00	0.00	0.00	0.00
4	1.92	2.74	0.31	0.32	0.19	0.15	0.00	0.00	0.00	0.00
5	2.17	3.05	0.38	0.40	0.20	0.18	0.00	0.00	0.00	0.00
6	3.28	3.92	0.49	0.46	0.38	0.07	0.00	0.00	0.00	0.00
7	4.44	4.91	0.66	0.58	0.24	0.05	0.00	0.00	0.00	0.00
8	5.28	6.00	0.86	0.77	0.15	0.06	0.00	0.00	0.00	0.00
9	3.58	7.06	0.61	0.90	0.54	0.03	0.00	0.00	0.00	0.00
10	6.64	7.32	1.12	1.14	0.13	0.11	0.00	0.00	0.00	0.00

C = Control cheese without the addition of bacterial protease T25.

P = Proteasea cheese with the addition of bacterial protease T25 (9.45 mg/L).

Table A-15: Change in Free Amino Acids (Isoleucine-Phenylalanine) in Citrate-HCl extract During Aging of Cheddar Cheese

Time (Months)	Free Amino Acid (µmol/g)									
	Isoleucine		Leucine		Lysine		Methionine		Phenylalanine	
	C	P	C	P	C	P	C	P	C	P
1	0.01	0.03	0.43	0.59	0.26	0.35	0.05	0.05	0.21	0.29
2	0.12	0.12	0.93	1.05	0.52	0.46	0.19	0.20	0.42	0.57
3	0.20	0.17	1.49	1.86	0.87	0.82	0.29	0.23	0.63	0.89
4	0.29	0.30	2.04	2.99	1.11	1.04	0.40	0.40	0.83	1.31
5	0.30	0.29	2.28	3.11	1.42	1.18	0.37	0.36	0.89	1.30
6	0.49	0.39	3.20	4.04	2.18	1.42	0.52	0.44	1.19	1.68
7	0.61	0.50	4.17	4.91	2.75	1.81	0.71	0.58	1.56	2.02
8	0.93	0.76	5.14	6.16	3.45	2.29	1.00	0.80	1.88	2.39
9	0.39	0.94	3.83	7.26	1.38	2.72	0.65	0.89	1.67	2.89
10	1.19	1.27	6.26	7.96	4.41	3.42	1.18	1.02	2.38	3.09

C = Control cheese without the addition of bacterial protease T25.

P = Protease cheese with the addition of bacterial protease T25 (9.45 mg/L).

Table A-16: Change in Free Amino Acids (Proline-Tryptophan) in Citrate-HCl extract During Aging of Cheddar Cheese

Time (Months)	Free Amino Acid (umol/g)									
	Proline		Serine		Taurine		Threonine		Tryptophan	
	C	P	C	P	C	P	C	P	C	P
1	0.11	0.27	0.02	0.09	0.09	0.09	0.01	0.03	0.00	0.13
2	0.29	0.25	0.16	0.26	0.43	0.35	0.10	0.13	0.00	0.00
3	0.39	0.46	0.20	0.21	0.19	0.13	0.16	0.15	0.13	[illegible]
4	0.45	0.51	0.33	0.30	0.19	0.15	0.27	0.43	0.22	0.2[illegible]
5	0.73	0.71	0.38	0.29	0.16	0.12	0.33	0.49	0.22	0.25
6	0.79	0.74	0.30	0.16	0.55	0.26	0.57	0.64	0.19	0.30
7	1.11	0.97	0.12	0.10	0.34	0.29	0.74	0.80	0.32	0.34
8	1.50	1.43	0.08	0.08	0.26	0.23	0.92	0.90	0.25	0.16
9	1.15	1.67	0.94	0.10	0.15	0.26	0.65	1.11	0.42	0.31
10	2.17	1.01	0.04	0.15	0.30	0.24	1.10	1.22	0.00	0.00

C = Control cheese without the addition of bacterial protease T25.

P = Protease cheese with the addition of bacterial protease T25 (9.45 mg/L).

Table A-17: Change in Free Amino Acids (Tyrosine-Valine) in Citrate-HCl extract During Aging of Cheddar Cheese

Time (Months)	Free Amino Acid (umol/g)			
	Tyrosine		Valine	
	C	P	C	P
1	0.06	0.10	0.27	0.26
2	0.08	0.12	0.38	0.42
3	0.06	0.05	0.60	0.73
4	0.06	0.09	1.00	1.11
5	0.12	0.08	1.05	1.18
6	0.10	0.07	1.55	1.53
7	0.13	0.15	2.06	2.04
8	0.14	0.13	2.52	2.41
9	0.55	0.16	1.63	2.98
10	0.07	0.17	3.31	3.49

C = Control cheese without the addition of bacterial protease T25.

P = Protease cheese with the addition of bacterial protease T25 (9.45 mg/L).

Table A-18: Ripening Index of Cheddar Cheese Samples

Age of Cheese (Months)	Soluble Tyrosine (mg/100 g of Cheese) Control	Protease (T25)
2	98.84	93.0
3	100.00	102.08
4	108.13	102.08
5	148.00	154.16
6	144.60	190.30
7	114.20	162.00
8	157.45	161.10
9	172.34	152.40
10	167.85	182.64

Data are mean values of duplicate determination for one lot of cheese.

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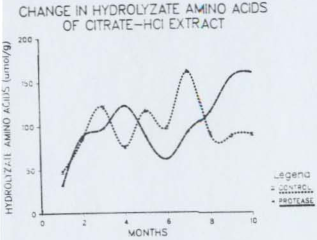

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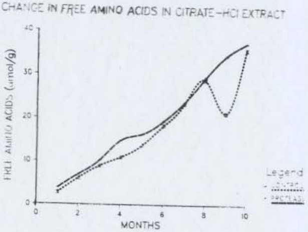

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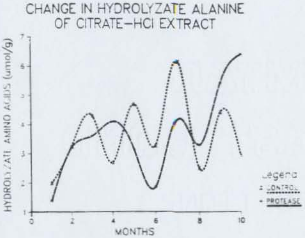

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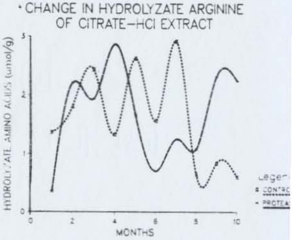

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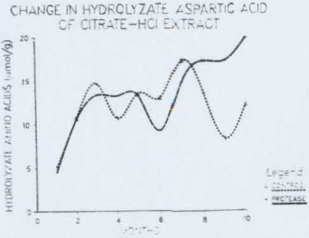

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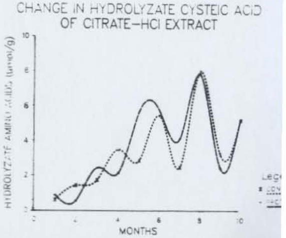

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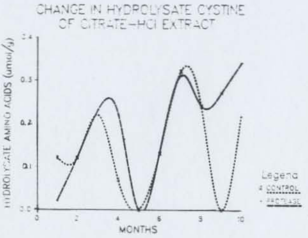

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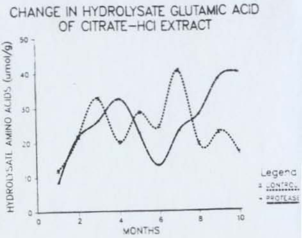

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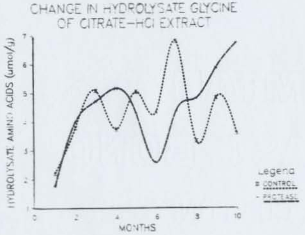

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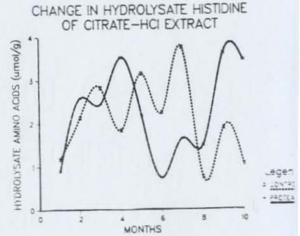

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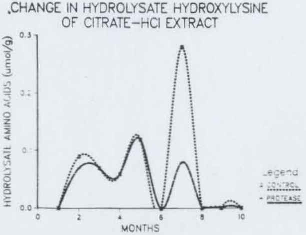

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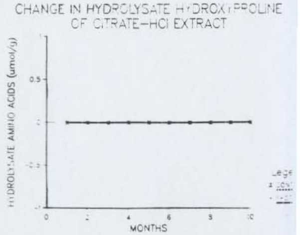

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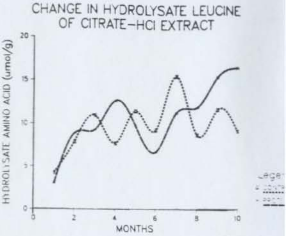
Figure A-14:



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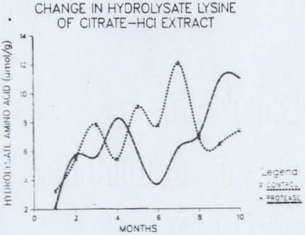

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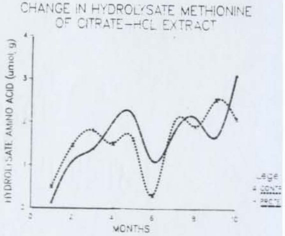

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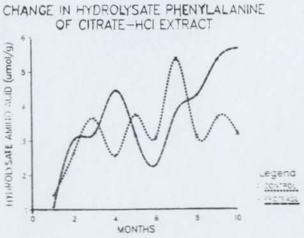

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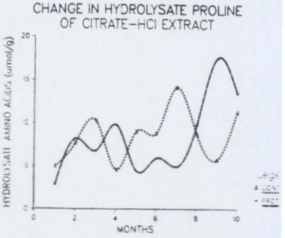

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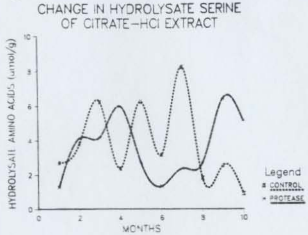

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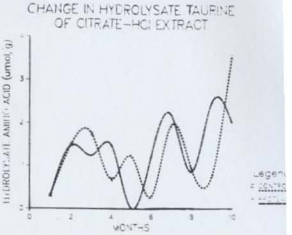

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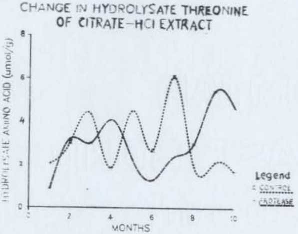

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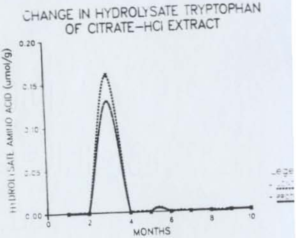

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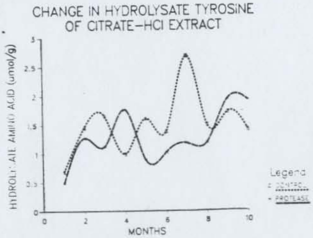

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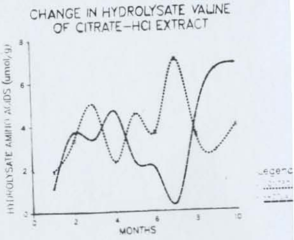

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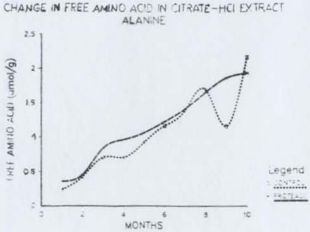

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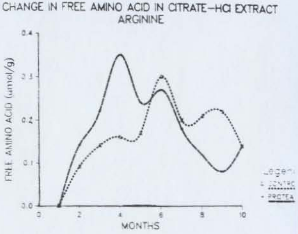

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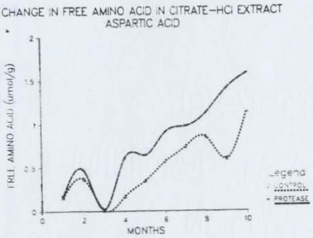

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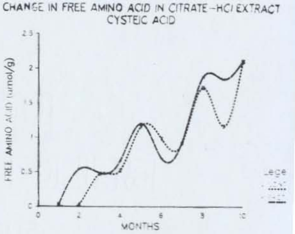

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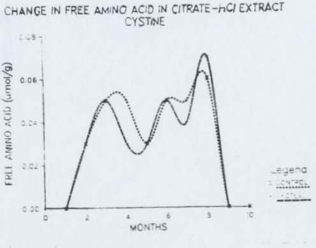

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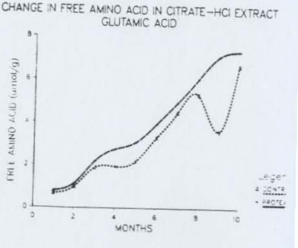

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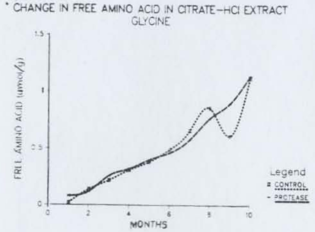

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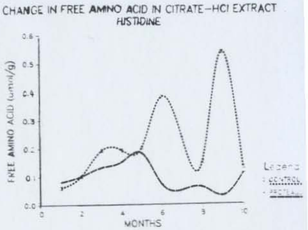

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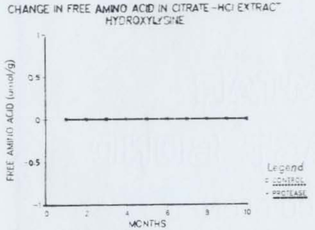

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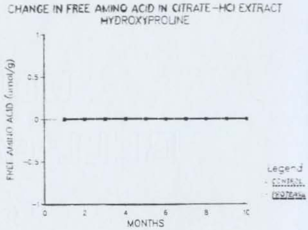

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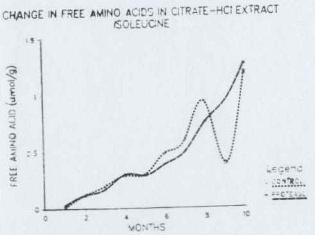

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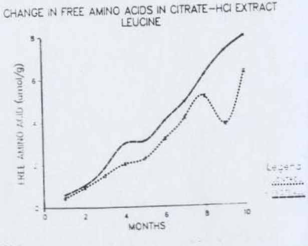

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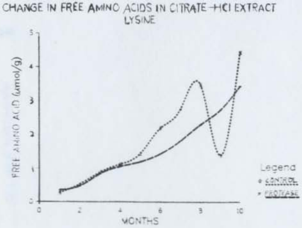

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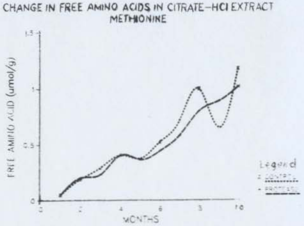

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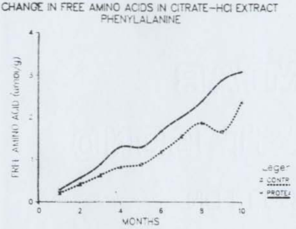

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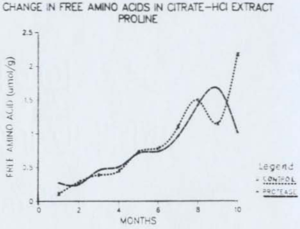

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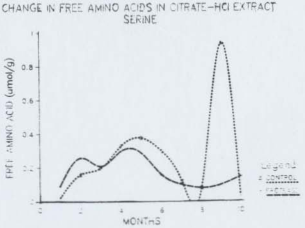

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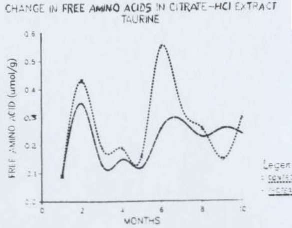

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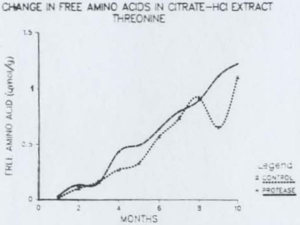

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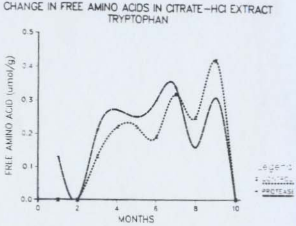

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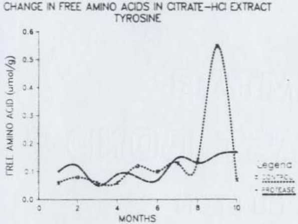

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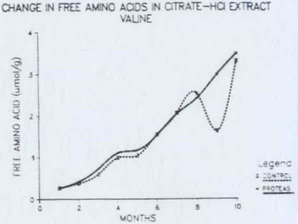
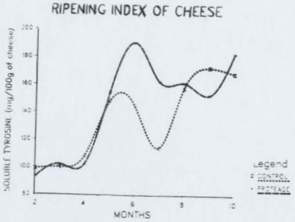


Figure A-47:



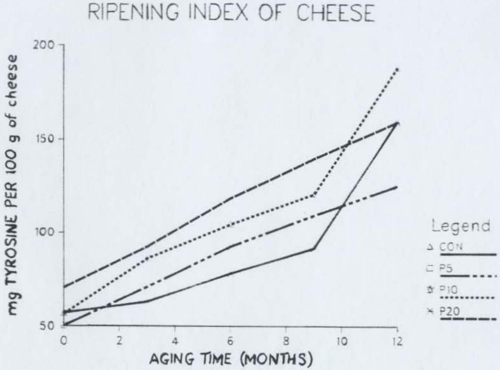


Figure A-48: Ripening index of experimental cheeses with different concentration of bacterial proteases (T25)

The above figure showed the effect of different concentration of bacterial proteases on the ripening index of experimental cheeses. The ripening index of cheese was determined by the method described by Vakaleris and Price (1959). They indicated that there was a trend in the relation between the age of cheese and the soluble tyrosine in the cheese extract. They also observed in their study that soluble tyrosine increased more rapidly in the early stages of ripening (Vakaleris and Price, 1959). In the above figure, CON represents the control cheese (without the addition of bacterial protease). P5 represents the protease treated cheese with the addition of 5 mg/L of bacterial protease T25. P10 indicates the concentration of 10 mg/L of T25 protease and P20 represents the concentration of 20 mg/L of T25 protease. In general, there was an increase in soluble tyrosine during the early stage of ripening. The three samples with added T25 protease almost consistently showed higher tyrosine level than the control (up to 9 months), in effect collaborating results of their effect in promoting cheddaring flavour.

Questionnaire for ranking

Name: ______________________ Date: ______________________

Rank these samples for the intensity of Cheddar cheese flavor. The sample with the sharpest Cheddar flavor is ranked first, the second sharpest samples is ranked two, and so on. Place the code numbers on the appropriate lines. These the samples in the following order: ___________.

____	____	____	____	____
1	2	3	4	5

Comments

QUESTIONNAIRE FOR SCORING

NAME: ____________________ DATE: ______________

Evaluate these samples for bitterness. Indicate the amount of bitterness in each sample on the scales below.

___ ___ ___ ___ ___

not bitter

----

trace of bitterness

----

slightly bitter

----

bitter

----

very bitter

----

extremely bitter

Comments;

Record of Manufacture

Type of Cheese ________

DATE ________

Maker					
Milk lbs.	Milk fat	%	lbs.		
Starter lbs.	Whey fat	%	lbs.		
Total lbs.	Starter acid	%	Age	hrs.	

Operation	Time	TEmperature	Acid %	pH	Comment	
Standardization						
Pasteurization						
Added starter					kind	%
Added color						
Added rennet					amount	
Added enzyme					amount	
Cutting						
Steam on						
Steam off						
Dipping						
Milling						
Salting						
Hooping						
Pressing						

Cheese	Date	Pounds	% Fat	% Water	pH	Yield after pressing