ISOLATION, PROPERTIES AND THE USE OF A CHYMOSIN-LIKE ENZYME FROM HARP SEAL (PAGOPHILUS GROENLANDICUS)

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Isolation, properties and the use of a chymosin-like enzyme from harp seal (*Pagophilus groenlandicus*)

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ABSTRACT

Four zymogens of acidic proteases A, B, C, and C' were isolated from the gastric mucosa of harp seal (Pagophilus groenlandicus) by ion exchange chromatography on a DEAE Sephadex A-50 column. Zymogens A and C were further purified by affinity chromatography using carbobenzoxy-D-phenylalanine-triethylene-tetramine and gel filtration on a Sephadex G-100 column. Certain physical and catalytic properties of proteases A and C were compared with those of calf chymosin (E.C.3.4.23.4) and porcine pepsin (E.C.3.4.23.1).

Zymogen C and the corresponding enzyme were homogeneous on analytical polyacrylamide gel electrophoresis. Zymogen A was homogeneous as judged by polyacrylamide gel elctrophoresis in the presence of sodium dodecyl sulphate and high performance liquid chromatography but was heterogenous as judged by polyacrylamide gel electrophoresis in the absence of SDS. Zymogens A and C had molecular weights of 26,300 ± 1540 and 37,100 ± 1075 daltons respectively as estimated by gel filtration but 33,800 + 1800 and 44,000 + 2100 daltons respectively as estimated by SDS polyacrylamide gel electrophoresis. Protease A had an isoelectric point of 4.90. The energies of activation of protease A and calf chymosin were 15.6 and 13.1 Kcal/mole calculated from the milk clotting activity of the enzymes.

Protease A was similar to calf chymosin with respect to several criteria. It had a higher ratio of milk clotting to proteolytic activity than those of seal protease C and porcine pepsin, clotted milk up to pH 7.0, and had a pH optimum of 2.2-3.5 for hemoglobin

hydrolysis. It did not inactivate ribonuclease, had very low activity on APDT and lost activity in 6M urea. These results indicate that protease A is chymosin-like rather than pepsin-like.

Experimental Cheddar cheese made with protease A, calf rennet, crude seal gastric protease (SGP) and Fromase (Mucor miehei protease) had comparable yields and proximate compositions. Sensory analysis of experimental cheeses after 30 weeks of aging showed that the product made with crude SGP had higher sensory scores than those made with the other coagulants. Chemical analyses showed more extensive degradation of protein in cheeses made with calf rennet and Fromase than in the cheeses made with the seal proteases. The electrophoretic patterns of the proteins from cheeses made with the seal proteases were qualitatively similar to those of calf rennet cheese. It was concluded that protease A and seal gastric proteases are promising as rennet substitutes.

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ABBREVIATIONS

APDT	N-acetyl-L-phenylalanyl-L-diiodotyrosine
BIS	N,N'-methylene-bis-acrylamide
DEAE-Sephadex	Diethylaminoethyl-Sephadex
EDTA	Ethylenediaminetetraacetic acid
RNA	Ribonucleic acid
SDS	Sodium dodecylsulphate
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
Z-D-phe-T-Sepharose	Carbobenzoxy-D-phenylalanyl-triethylene-tetramine-Sepharose

INTRODUCTION

Preliminary studies indicated that a crude extract from the stomachs of harp seal (Pagophilus groenlandiscus) pups had properties similar to those of calf chymosin and different from most pepsins. The crude extract had milk clotting activity similar to that of calf chymosin in the pH range 6.0 to 6.7, and different from that of porcine pepsin, had a higher ratio of milk clotting activity to hemoglobin hydrolytic activity than that of pepsin, was more stable than pepsin in casein at pH 6.1 and had a higher pH optimum for hemoglobin hydrolysis than that of pepsin (see Appendices A-D). Cheddar cheese prepared with the crude extract was comparable to that made with calf rennet in yields and sensory qualities (Appendix E). These observations led to the hypothesis that crude SGP contains a chymosin-like enzyme. Occurrence of a chymosin-like enzyme in rat stomach has been reported by Kotts and Jenness (1976) based on the pH optimum of the gastric juice for hemoglobin hydrolysis, although chymosin is believed to occur only in young ruminant stomachs (Foltmann, 1970). Malpress (1967) demonstrated the absence of any protease that was stable in alkaline buffer in the gastric juice of children and concluded that chymosin does not occur in human stomach. However, Hirsh-Marie et al. (1976) reported the presence of a foetal pepsinogen that did not cross react with antibody against adult human pepsinogen. Therefore, occurrence of chymosin in non-ruminant stomach is still a controversy.

The possible existence of the multiple isoenzyme forms of seal gastric proteases was also of interest, in view of the occurrence of

several isomers of gastric proteases in other species including humans (Asato and Rand, 1977; Kageyama and Takahashi, 1976; Meitner and Kassel, 1971; Ryle, 1970; Donta and Van Vunakis, 1970; Bar-Eli and Merrett, 1970; Etherington and Taylor, 1970). Partial amino acid sequence of a "pepsinogen" from ring seal (Phoca hispida) has been reported by Klem et al., (1976). However, the enzyme was not characterised to examine whether it is a pepsin, chymosin, or gastricsin. The possible occurrence of chymosin and of multiple isoenzyme forms was also not addressed. For these reasons gastric proteases from another pinniped species Pagophilus groenlandicus were studied. Harp seal stomach was chosen because it is easily available as a by product from the seal fishery in Newfoundland.

Preparation of Cheddar cheese was also repeated using the crude SGP and protease A. Cheeses were also prepared with calf rennet as control and Fromase (Mucor miehei protease), a leading commercial rennet substitute, for comparison.

BACKGROUND

The proteolytic enzyme traditionally used as milk coagulant for cheese making is rennet - the crude preparation from suckling calf stomach containing mainly chymosin (E.C.3.4.23.4). However, during the last twenty years there has been a shortage of calf rennet because of an increasing production of cheese and decrease in the number of young calves slaughtered (USDA, 1972). This shortage generated considerable interest in a search for new sources of milk coagulants. Proteolytic enzymes from plants, animal organs, and microorganisms have been extensively studied (see Ernstrom, 1974; Green, 1977 for review). A small number of rennet substitutes are now in commercial use. In 1976 calf rennet supplied only 25% of the world demand for milk coagulants (deKoning, 1978). Milk coagulating enzymes from various sources and their characteristics will be briefly discussed here.

1. Chymosin

Chymosin is an acidic protease secreted by the abomasa of young ruminants. It is secreted as a precursor called prochymosin. Calf prochymosin has a molecular weight of 36,000 daltons. It undergoes autoactivation optimally at pH 2.0. The activation involves removal of a small peptide bond from the aminoterminus of the precursor (Foltmann, 1970). The isoelectric point of calf chymosin is 4.7 (Righetti et al., 1977). The primary structure of calf chymosin has been elucidated (Foltmann et al., 1979).

Chymosin possesses some unique characteristics that make it the

enzyme of choice for cheese production. During milk clotting, it is highly specific for a particular peptide bond in κ-casein (Delfour et al., 1965) under the conditions used in cheese making. As a result of this narrow specificity there is minimum solubilization and loss of curd protein resulting from proteolytic action. The enzyme is relatively stable at the pH of the curd so that the residual enzyme carried into the cheese contributes to flavor development by proteolysis during aging. The rate and specificity of proteolysis by chymosin during the ripening stage are conducive to good texture and flavor of the aged cheese. The narrow specificity of chymosin is also evident from its lack of action on ribonuclease (Bang-Jensen et al., 1964), which is hydrolysed by pepsin (Berger et al., 1959). Moreover, the number of chymosin-susceptible bonds in the B chain of insulin is less than the number of pepsin-susceptible bonds (Bang-Jensen et al., 1964). Chymosin has a higher ratio of milk clotting to proteolytic activities (Green, 1972) and higher pH optimum for protein hydrolysis than does pepsin (Foltmann, 1959; Chiang et al., 1967). Chymosin is also more stable than pepsin under conditions of milk clotting, curd handling and cheese ripening (Green and Foster, 1974). During ripening of cheese, chymosin preferentially hydrolyses α_s-casein (Ledford et al., 1966).

Foltmann (1970) separated calf chymosin into three different fractions having minor differences in their properties. Chymosin or crude preparations containing chymosin called rennet from other ruminants have also been studied. Lamb chymosin was chromatographically separated into three different fractions which had different ratios of milk clotting to proteolytic activities (Oruntaeva and

Seitov, 1971). Anifantakis and Green (1980) examined kid and lamb rennet. They found that rennets from these animals have lower ratios of milk clotting to proteolytic activities than calf rennet. Qadri et al. (1962) isolated "rennet" from adult goats. The yield of "rennet" was low compared to calf rennet. Lamb rennet has been reported to be more efficient in clotting sheep milk than bovine milk (Herían and Krcal, 1971).

2. Rennet substitutes

a) Pepsins

Pepsin (E.C.3.4.23.1) is similar to chymosin in many respects. Both are acidic proteases secreted by the animal stomach and auto-activated in the acidic medium of the organ as a result of the removal of a small peptide from the amino terminus of the corresponding precursors (Foltmann, 1970; Ryle, 1970). Certain sections of the polypeptide chain of chymosin and pepsin have been found to have similar amino acid sequences. However, chymosin was reported to be immunologically distinct from pepsin (Rothe et al., 1976). Pepsin has been widely studied as a rennet substitute perhaps because of the similarities between chymosin and pepsin discussed above and the fact that commercial rennet used for cheese production always contains some amount of pepsin (Emmons et al., 1978; Emmons et al., 1976). Reports on the suitability of bovine pepsin as a rennet substitute are contradictory. Bottazzi et al. (1974), Corradini et al. (1974) and Phelan (1973) reported successful use of bovine pepsin in cheese production. Fox and Walley (1971) reported no significant difference between the cheeses made with bovine pepsin and those made with calf

rennet either alone or in a 50:50 blend. However, the pH 4.6 soluble N in aged cheese made with 100% bovine pepsin was lower than that of the cheese made with rennet indicating slower rate of protein degradation in cheese made with 100% bovine pepsin than in rennet cheese. Fox (1969) showed that bovine pepsin coagulates milk at pH 6.9. This observation suggests that the enzyme is stable at this pH and presumably also at other stages of cheese making and ripening.

Green (1972) found that the ratio of milk-clotting to proteolytic activity of bovine pepsin was slightly higher than that of calf rennet when tested on hemoglobin and slightly lower when based on casein as substrate. However, purified bovine pepsin has a lower ratio of clotting to proteolytic activity than calf chymosin (Amer et al., 1980; deKoning, 1978). The Cheddar cheese made using bovine pepsin was slightly inferior to that made with calf rennet. Emmons et al., (1978), Emmons et al., (1976), Phelan (1973) and Stanley and Emmons (1977) have confirmed this observation by reporting that bovine pepsin was associated with slightly reduced yield and lower quality of Cheddar cheese.

Bovine pepsinogen can be separated chromatographically into four components. All these fractions have potential hydrolytic activities on hemoglobin and also on the synthetic substrate N-acetyl-L-phenylalanyl-L-diiodotyrosine (APDT) according to Kassel and Meitner (1970). Foltmann et al., (1979) showed considerable homology in the amino acid sequences of porcine pepsinogen and bovine prochymosin.

Use of porcine pepsin as a rennet substitute dates back to the earlier part of the twentieth century (See Ernstrom, 1974 for a review). Studies on the use of porcine pepsin as a rennet substitute

were intensified during the last two decades (Chapman and Burnett, 1968; Melachouris and Tuckey, 1964; Emmons et al., 1962; Olson, 1971; Thomasow, 1971; O'Keefe et al., 1977). There are conflicting reports on the quality of cheese prepared with porcine pepsin. Davies et al., (1934) reported bitterness in Cheddar cheese made with porcine pepsin. Maragoudakis et al. (1961) and Melachouris and Tuckey (1964) showed that there was no bitterness in Cheddar cheese made with porcine pepsin.

However, there are difficulties in using porcine pepsin as a rennet substitute. Milk setting takes longer, and results in softer curd and loss of fat (Emmons et al., 1970; Chapman and Burnett, 1968). The longer setting time is attributed to inactivation of the enzyme above pH 6.0 (Ryle, 1970). Green (1972) and Green and Foster (1974) showed that during the normal Cheddar cheese making process all or most of the porcine pepsin is destroyed. This report is consistent with the observation by Melachouris and Tuckey (1964) that Cheddar cheese made with porcine pepsin ripened slowly.

Emmons et al., (1971) used a 1:1 mixture of calf rennet and porcine pepsin in Cheddar cheese manufacture; they found that the cheese made with the mixture and with control rennet were essentially of equal quality. They also suggested that porcine pepsin should not be used singly, because slow milk setting and fat loss into whey might present problems. The research committee of the National Cheese Institute (1960) recommended that porcine pepsin should not be used alone in cheese manufacture.

Emmons (1970) observed that porcine pepsin is quickly inactivated by the high pH of hard water at the ambient temperature used in cheese

plants. Ernstrom (1961) showed that between pH 6.4 and 6.7 porcine pepsin had much longer milk clotting times than equal milk-clotting potency of calf chymosin at pH 6.3. This suggests that porcine pepsin is relatively unstable under these conditions. Mickelsen and Ernstrom (1972) found porcine pepsin to be unstable above pH 6.0. Cheese made with porcine pepsin has a bland taste (Sardinas, 1972) because the enzyme is inactivated during cheese making (Green, 1972).

Like bovine pepsin, porcine pepsin exists in multiple isoenzyme forms. Ryle (1966) separated crude porcine pepsin into a major component, A, and minor components B, C and D. They are all believed to be products of separate pepsinogens. Pepsins B and C are stable up to pH 6.9 but clot milk less readily than pepsin A (Ryle and Porter, 1959). Crude porcine pepsin contains a gastricsin, another gastric proteolytic enzyme (Chiang et al., 1967). Pepsin and gastricsin are differentiated by their substrate specificity. Pepsin can readily hydrolyse the synthetic substrate N-acetyl-L-phenylalanyl-L-diiodotyrosine (APDT) whereas gastricsin cannot (Tang, 1970). The pH optimum for hemoglobin hydrolysis by porcine gastricsin is 3.0, while that of pepsin is 2.0 (Chiang et al., 1967). The pH stability of gastricsin is similar to that of pepsin and is probably subject to the same stability problems as porcine pepsin during cheese manufacture (Ernstrom, 1974). However, fish gastricsins are relatively stable in alkaline pH (Chiang and Ponce, 1981).

Chicken pepsin was used in laboratory scale Khashkhaval and Emmental cheese preparation in Israel (Gordin and Rosenthal, 1978). There was no appreciable difference in taste, flavor, or texture between the cheeses made with chicken pepsin and those prepared with

calf rennet. Neither of the cheeses had any bitterness. While the soluble-nitrogen and free amino acid contents of the Emmental type cheeses were comparable in both the cases Khashkhaval cheese made with chicken pepsin showed much higher soluble nitrogen than rennet cheese suggesting enhanced proteolysis in the Khashkhaval cheese made with chicken pepsin. The authors also report the use of chicken pepsin for the successful manufacture of many kinds of cheeses in Israel.

Green (1972) reported that a Cheddar cheese trial with chicken pepsin resulted in poor quality cheese with texture and flavor defects, probably due to excessive proteolysis by residual chicken pepsin. Stanley et al., (1980) also reported chicken pepsin to be unsuitable for Cheddar cheese because the cheese made with it resulted in softer body and bitter taste. In addition, there aws an increased loss of nonprotein nitrogen(NPN) into whey from curd made with chicken pepsin, compared to that made with calf rennet.This increased NPN loss suggests a potentially lower yield of cheese than the cheese made with calf rennet. Nonprotein nitrogen in the cheese made with chicken pepsin was approximately twice as much as in the cheese made with calf rennet. Donta and Van Vunakis(1970) characterized chicken pepsin.Chicken fore-stomach 4 different pepsinogens.Pepsinogens A and D and their corresponding enzymes were similar in amino acid composition.Pepsinogen C was immunologically different from pepsinogens A and D.Pepsinogen B was active only on the synthetic substrate N-Cbz-L-Glu-L-Tyr.Bohak(1970) showed that chicken pepsin was stable between p^H 1.0 and 8.0 at 25°C.

(b) Microbial rennet substitutes

Microorganisms are probably the best means of producing low cost

enzymes having uniform quality. There are thousands of genera to choose from, and it is relatively easy to control production of microbial enzymes. Numerous genera of microorganisms have been screened for production of suitable rennet substitute (Tendler and Burkholder, 1961; Srinivasan et al., 1964; Schulz et al., 1967; Arima et al., 1967; Sardinas, 1968; Prins and Nielsen, 1970). However, only a small number of organisms have been found to be promising. Limited success may be attributed to broad substrate specificity of microbial proteases in general (Hagihara, 1960).

Sardinas (1972) reviewed the literature on bacteria reported to produce milk-clotting enzymes including the genera Alkaligenes, Bacillus, Corynebacterium, Escherichia, Lactobacillus, Proteus, Pseudomonas, Serratia, Staphylococcus, Streptococcus, Streptomyces, Thermoactinomyces, and Vibrio. Of these, the genus Bacillus has been thoroughly studied. A heat-labile alkaline protease from Bacillus polymyxa was patented as a rennet substitute (Godo Shusei Co., 1968, 1969; Imai et al., 1970). However, cheesemaking trials showed a small but distinct loss in curd yield. These authors reported no bitter flavor of the cheese after 4 months of aging, others have observed bitter flavor in the curd made with this enzyme preparation (Emmons and Elliott, 1972).

Bacillus cereus protease was patented as a rennet-substitute by Miles Laboratories Inc. (Sardinas, 1969). Cheddar cheese made with this enzyme was found to be free of bitter flavor (Srinivasan et al., 1962a, b). However, the texture was not typical of Cheddar cheese and maturation was delayed. Melachouris and Tuckey (1968) reported higher proteolytic action of this enzyme than rennet on casein. The enzyme

hydrolysed β-casein to a greater extent than did calf rennet.

Patents were issued to Murray and Prince (1970) for making cheese with rennet substitute from Bacillus subtilis. These proteases were found to release much more NPN from milk than did calf rennet at the clotting level and to degrade α and β caseins more rapidly and nonspecifically (Puhan, 1969). Bacillus subtilis milk coagulant has not been used commercially.

Numerous genera of fungi also have been screened for suitable proteolytic enzymes, and patents have been granted for some of these enzymes (See Sardinas, 1972 for a review). Whitehill et al. (1960) and Oringer (1960) were granted patents for commercial preparation of milk clotting enzymes from Conidiobolus, Entomopthora, and Basidioleolus. The enzymes were characterised and found to result in bitter flavor in cheese.

Proteolytic enzymes from Endothia parasitica, Mucor pusilus (Lindt) and Mucor miehei are being commercially used. Sardinas (1965, 1967, 1968), Larson and Whitaker (1970) and Hagemeyer et al. (1968) characterised the acidic protease from Endothia parasitica. It has a molecular weight of 34,000 to 39,000, and an isoelectric point of pH 4.6. The milk clotting activity of the enzyme was less sensitive to pH change in the range 5.1 to 6.5 than calf rennet.

It was found to have broader substrate specificity than chymosin and pepsin. The enzyme has been reported to accelerate the ripening of Cheddar cheese (Shovers and Bavisotto, 1967). Cheddar cheese prepared using a 3:1 mixture of the enzyme with calf rennet developed poor flavor and texture (Morris and McKenzie, 1970). Endothia parasitica protease was found to be more proteolytic than calf rennet

and produced bitterness in Edam, Tilsit and Butter cheeses (Thomasow et al., 1970). Ramet et al., (1969) reported the preparation of good quality Emmental cheese with protease from E. parasitica. Production of Emmental cheese involves cooking at high temperature. The enzyme is probably inactivated at that temperature before it effects hydrolysis to produce bitter peptides.

An acidic protease from Mucor pusilus (Lindt) was characterized by Arima et al., (1967) and Iwasaki et al., (1967a, b, c), and its properties were later reviewed by Arima et al., (1970). The enzyme has an isoelectric point of pH 3.5 to 3.8 and is stable in the pH range 4.0 to 6.0. The proteolytic activity of the enzyme is less specific than that of calf rennet. It was found to be more proteolytic than calf rennet, destroying β-casein to a greater extent than did calf rennet and pepsin (Mickelsen and Fish, 1970). Mucor pusilus protease was found to be a satisfactory rennet substitute in the manufacture of Brick, Butter, Camembert, Cheddar, Cottage, Edam, Gouda, some Italian varieties and Tilsit cheeses (Arima et al., 1970; Schulz et al., 1967). However, bitter flavor was reported in some cheeses made with the enzyme (Richardson et al., 1967; Kikuchi and Toyoda, 1970). Richardson et al. reported that MP protease was incompatible with calf rennet, because in solution it destroys the latter.

Patents have been granted for manufacture of milk coagulant from several strains of M. Miehei. These are M. miehei Cooney et Emerson (Aunstrup, 1968), M. miehei NRRL 3420, M. miehei ATCC 1645.7 (Feldman, 1969), M. miehei NRRL 3169 (Baxter Laboratories, Inc., 1970). Several companies are selling these milk coagulants under various trade names (Rennilase - Novo Industries, Fromase - G.B. Fermentation Industries, Ill.)

Proteolytic enzymes from M. miehei Cooney et Emerson was purified and characterised by Ottensen and Rickert (1970a, b). Like the other two commercial fungal rennets, M. miehei rennet is an acidic protease with an optimum pH for hemoglobin hydrolysis at 4.5. Its specificity on the oxidized B chain of insulin was reported to be similar to that of calf rennet. Ottensen and Rickert (1970b) observed that the enzyme is quite stable; it retained 90% of its activity in the pH range 3 to 6 during 8 days of incubation at ambient temperature. It has a molecular weight of 38,000 daltons and an isoelectric point of pH 4.2.

Yield and quality of Emmental cheese manufactured with M. miehei protease were the same as that with calf rennet, and there was no bitter taste (Kiuru, 1969; Mayr, 1971). Thomasow et al. (1971) made similar observations about Edam, Tilsit and Butter cheeses made with M. miehei rennet. Domiati cheese made with M. miehei rennet was satisfactory, although it contained more soluble N than calf rennet cheese (Hamdy, 1970).

(c) Rennet Substitutes from higher plants.

Rennet substitutes from higher plants were sought not only because of rennet shortage but for religious and ethnic reasons as well (Reed, 1975). In India, enzymes from fig and a wild shrub (Strebius asper) yielded slightly less curd than that made with calf rennet (Veringa, 1961). Both curds were softer than that made with calf rennet. According to Oosthuizen and Scott Blair (1963) the mechanism of casein hydrolysis by ficin is different from that of calf rennet. Properties of ficin, a sulphydryl proteolytic enzyme from fig have been reviewed by Liener and Friedensen (1970). The milk clotting and proteolytic activities of ficin have been reported to be

due to two different factors. Cheese made with ficin had bitter flavor which disappeared with aging, presumably due to degradation of bitter peptides (Krishnaswamy et al., 1961).

Bromelain, a proteolytic enzyme preparation from pineapple was reported to be too proteolytic to be used as a rennet substitute (Poszarne-Hanjal et al.,1969). Papain from papaya latex was reported to have strong proteolytic activity (Dastur, 1948) but its proteolytic activity was also too high for use as a rennet substitute. Papain has a free sulphydryl group essential for catalytic activity and loses activity reversibly due to oxidation in air in the absence of added cysteine (see review by Arnon, 1970). There is controversy over the catalytic activities of papain, one group of workers supporting the view that proteolytic and milk clotting activities are the same whereas another group believes these two properties are unrelated (Skelton, 1971; Hinkle and Alford, 1951; Balls and Hoover, 1937).

Kothavalla and Khubchandani (1940) used extract from Withania coagulans for making some Indian varieties of cheese in which no unusual flavor was found. However, there were high fat losses. Extract from cardoon flower is traditionally used by Portuguese farmers for Serra cheese from sheep's milk. Studies on the milk clotting properties of the extract showed that the clotting activity was more sensitive to pH of milk than calf rennet (Vieira de Sa and Barbosa, 1970a, b, c). It was more proteolytic than calf rennet when used in Edam cheese which was bitter and acid. Ernstrom (1974) reported extremely bitter flavor in Cheddar cheese made with cardoon extract only after 30 days of ripening. Gupta and Eskin (1977) isolated a milk coagulant from ash gourd and used it for Cheddar

cheese. It was found to be a neutral protease. Cheddar cheese made with the ash gourd protease had a slightly lower sensory score than that of rennet cheese. Fat loss in whey was higher and the yield of cheese was lower.

3. Conservation of rennet and other alternatives

Shortage of rennet may also be circumvented by making changes in the conventional method of cheese production. The amount of coagulant required for making cheese may be reduced by (1) concentrating the milk, (2) using $CaCl_2$, (3) raising the milk temperature, and (4) the coagulant may be immobilized for repeated use.

The use of twofold concentrated milk to make Cheddar and Cheshire cheeses resulted in 75-80% saving of rennet compared with the conventional method. However, both the cheeses showed less intense flavor than the cheeses made with untreated milk (Chapman et al., 1974). The above-named authors also used milk concentrated more than twofold to make Cheddar cheese. They found that the cheeses so prepared were of poor quality (Chapman et al., 1979). Green et al. (1981) prepared Cheddar cheeses with 1.7-to 4-fold concentrated milk and found that the fat contents of the cheeses were less than normal. Moreover, with increase in the concentration factor of the milk, the rate of casein breakdown, the intensity of Cheddar flavor and the concentrations of H_2S and methanethiol in the cheeses decreased.

Addition of $CaCl_2$ to milk in order to reduce the amount of coagulant required to clot milk is a common practice, particularly with the more proteolytic coagulants (Green, 1977). However, the use of $CaCl_2$ is reported to reduce the ripening rate of cheese (Ernstrom

et al., 1958).

Other conservation methods include the recovery of rennet from whey (Green, 1977). This method would have an additional advantage in that the rennet free whey is more suitable for the preparation of concentrated whey protein. Another method is the possible use of fistulated calves to produce rennet continuously. Ganguli (1970) reported that such calves produce rennet at a satisfactory rate.

The production of microbial rennets by genetically "enhanced" microorganisms has been suggested by Welsey (1981). However, since the commercial rennets of microbial origin are not completely satisfactory in terms of the yield and quality of the cheese they produce (deKoning, 1978) an ideal solution to the rennet shortage would be to mass produce calf chymosin by incorporating the calf chymosin genes into appropriate microorganisms. Techniques are now available for the isolation of eukaryotic genes and their incorporation into microorganisms (Dally et al., 1981). The

The use of immobilized proteases is another possible choice to conserve rennet.Green and Crutchfield(1969) immobilized calf chymosin to use it repeatedly for clotting milk.However, the enzyme had low activity because of inactivation during the immobilization and because of leaching of the enzyme from the support.Successful immobilization of chymosin may only partly solve the problem of rennet availability, because the curd formed by the immobilized enzyme would lack the residual chymosin required for the ripening process of the cheese.However, rennet can be added to the curd and there would still be a net saving of rennet.The successful immobilization of any protease which does not affect the yield of curd should be suitable for this purpose. Ohmiya et. al.,(1979)

immobilized calf rennet and an alkaline protease from Bacillus subtilis on Dowex. Rennet was found to have a very short half-life, whereas the alkaline protease from B. subtilis had a halflife of 8 days after immobilization. Cheddar cheese prepared by using the immobilized alkaline protease was found to ripen at a rate similar to normal rennet cheese. The organoleptic quality of this cheese was similar to those of normal and immobilized rennet cheeses. However, the ripening rate as judged by the release of NPN for immobilized calf rennet was slower than that of normal calf rennet cheese. These studies cast doubt on the supposition that residual rennet contributes to flavor development. Green (1977) has argued that the interpretation of these studies is dependent on the investigator carefully establishing that no enzymes leach from the reactor into the milk.

4. Chemistry of milk clotting

Enzymatic milk clotting occurs in two phases. In the primary or enzymatic phase, κ-casein in milk undergoes hydrolysis and thus loses its ability to stabilize the casein micelle. In the secondary or nonenzymatic phase the micelles aggregate to form a clot. This takes place in presence of Ca^{++} and the mechanism by which it brings about the aggregation is not fully understood. Alais et al., (1953) has demonstrated this biphasic nature of milk clotting. The enzymatic reaction was carried out at 2°C at which no clotting occurred. Coagulation took place immediately on warming the milk to a higher temperature. Berridge (1942) made a similar observation. Dalgleish (1979) showed that coagulation is not initiated until 88% of the κ-casein is hydrolysed. The action of chymosin on κ-casein was the

subject of extensive studies (Wake, 1959; Garnier, 1958) until Delfour et al., (1965) established that chymosin hydrolyses a peptide bond involving phenylalanine and methionine. As a result a soluble glycomacropeptide and an insoluble peptide are produced. The glycomacropeptide is a heterogeneous glycopeptide with a molecular weight of 6,000 to 8,000 daltons (Hill et al., 1970; Nitschmann and Henzi, 1959). Besides chymosin other proteases also, e.g. pepsin and chymotrypsin (Dennis and Wake, 1965; Green, 1972), E.parasitica protease (Vanderpoorten and Weckx, 1972), M. pusilus protease (Yu et al., 1968) also attack κ-casein at the same site.

The secondary phase of milk clotting is not clearly understood because it is not known how the major casein components, namely α, and β caseins interact with each other and also with κ-casein (which is apparently responsible for micelle stability) and colloidal calcium phosphate to form a stable suspension called casein micelle. Nor is it known how κ-casein loses its capacity to stabilize the micelle because of hydrolysis. The function of Ca^{++} to bring about aggregation of the micelles is also not clearly understood. There are various models to explain the micellar arrangement of caseins in milk and the mechanism of enzymatic milk coagulations but none of them satisfactorily explains clotting phenomenon (see Ernstrom, 1974 for a review). Most recent model of casein micelle is based on reconstitution of micelle using gold labelled casein components (Payens, 1981). Electron micrograph of casein micelles reconstituted using gold labelled κ-casein showed that all the κ-casein is on the surface. Similar experiments using labelled α and β caseins showed that these components are in the core of the reconstituted micelle. However, Payens (1981)

did not mention whether the micelles thus reconstituted behave like the native casein micelles in milk clotting.

5. Criteria for a good rennet substitute

Zwaginga and Nandts (1973) recommended that a coagulant should have the following properties to replace calf rennet: (a) yield of cheese should be equal to that made with calf rennet; (b) cheese should not develop off-flavor or bad texture; (c) the use of the coagulant should not require any change in standard cheese-making practices; (d) should be free from toxic and antibiotic activity and pathogens; (e) should not have lipolytic or other contaminating enzymes; (f) should not be too proteolytic; and (g) price should be acceptable.

According to deKoning (1978) none of the commercially available rennet substitutes meet all the requirements listed above. For example, pilot-scale trial with porcine pepsin resulted in lower quality Cheddar cheese. Although the yields of Cheddar cheese made with bovine pepsin and enzymes from Endothia parasitica, Mucor pusilus and Mucor miehei were lower, the cheeses were otherwise satisfactory. All the commercial fungal rennets contain amylase.

The most common problem with rennet substitutes is the development of flavor and texture defects in cheese (deKoning, 1978). This is attributed to high proteolytic activity of most rennet substitutes relative to their milk-clotting activity (Green, 1977). Excessive proteolysis also results in loss of fat in whey (Veringa, 1961). Too little proteolysis by the residual coagulant also has undesirable effects on flavor. For example, Cheddar cheese made with porcine

pepsin failed to develop characteristic aged flavor(Green and Foster, 1974).

Proteolytic specificity is another important factor. Degradation of α_s-casein is predominant in a normally ripened cheese(Ledford et. al., 1966; Stanley and Emmons, 1977) whereas the cheeses with poor flavor are associated with a high rate of degradation of β-casein(Phelan et. al., 1973). The ratio of milk-clotting to proteolytic activities of a rennet substitute is a generalyy accepted indication of its narrow specificity (Green, 1972). However, the best way to determine if a proteolytic enzyme is a good rennet substitute is to prepare cheese on an industrial scale (deKoning, 1978).

Lipase, associated with microbial rennets (Richardson et. al., 1967) is detrimental to the flavor of certain cheeses(Green, 1977). Some Italian cheeses require pregastric lipase present in rennet paste which cannot be repalced by any other lipases(Long and Harper, 1956).

6. Flavor, texture and cheese-ripening

The development of flavor in a cheese with aging is a function of the breakdown of carbohydrate, fat and protein by the enzymes from the coagulant and the microorganisms growing and decaying in the cheese. The combined action of these enzymes result in the production of a few classes of compounds. Some of these compounds, e.g. amino acids and carbonyls are qualitatively common to all the cheese-types while others are characteristic of a particular cheese-variety. Harper(1959) separated these compounds broadly into two categories by vacuum distillation. The volatile distillate contributes aroma while the non-volatile residue imparts the sensation of taste to the cheese.He showed

that the non-volatile part was composed of lactic acid, amino acids, keto acids and other non-volatile acids and amines and salt. The volatile component is composed of fatty acids, aldehydes, ketones, alcohols, amines, esters, H_2S and sulphides.

An examination of the literature on cheese flavor shows that the relationship between characteristic flavor of cheese and a chemical compound or a group of compounds is based on (a) the presence in the cheese of that compound, (b) increase in concentration of the compound with ripening and increase in flavor intensity of the cheese, and (c) reproduction of the cheese flavor when the compound in question is added to a bland base. However, one has to be cautious in drawing conclusions using such criteria. A compound found to be associated with flavor may actually have been produced in parallel with another compound which is directly responsible for the flavor. Also the flavor compound may be present in chemically undetectable amounts. The failure of an added compound to reproduce or to enhance a particular aspect of flavor does not rule out the compound as a flavor component because it is almost impossible to add the compound in its original chemical and physical form along with other compounds which may play supplementary roles in expression of the flavor.

Harper (1959) observed a definite correlation between free amino acids and characteristic Cheddar cheese flavor. Cheese with low free amino acid content lacked flavor and cheese with high amino acid content had characteristic flavor. Glutamic acid occurred in the highest concentration and appeared to have a direct correlation with flavor. Furthermore, Harper found that free amino acids added to a bland base in concentration found in Cheddar cheese resulted in cheese

flavor lacking aromatic character. Silverman and Kosikowski (1953) made similar observations with Swiss cheese flavor. Others also reported occurrence of free amino acids in cheese (Harper and Swanson, 1949; Kosikowski, 1951; Lindqvist et al., 1953; Bullock and Irvine, 1956; Melachouris and Tuckey, 1964). O'Keefe et al., (1976) stated that the full range of amino acids found in casein also occur in cheese and their liberation is accompanied by flavor development. O'Keefe et al., (1976) observed that only a limited range of free amino acids, namely methionine, histidine, glycine, serine and glutamic acid are liberated in measurable amounts in cheese which were chemically acidulated instead of adding starter culture before renneting. This observation led to the conclusion that free amino acids in Cheddar cheese are mainly the result of microbial peptidase activity. Salji and Kroger (1981) observed increase in peptidase activity in Cheddar cheese with ripening.

Mabbit (1961) demonstrated that the ratios of the quantities of free amino acids occurring in Cheddar cheese were in some instances different from those in casein. He added an aqueous solution of a mixture of amino acids in proportion found in six-month-old Cheddar cheese to freshly made curd. Sensory evaluation of this "cheese" had a brothy flavor but no Cheddar flavor. Dacre (1953) also made a similar observation. However, Mabbit (1961) concluded from his studies that the free amino acids contribute towards the background flavor.

There seems to be lack of agreement between workers on the relative contribution of microorganisms and coagulant enzymes towards

proteolysis in cheese. Orla-Jensen (1939) showed that rennet alone can hydrolyse milk protein but cannot produce free amino acids. O'Keefe et al.,(1976) concluded from their observations on Cheddar cheese made with and without starter organisms that microorganisms make no significant contribution to the formation of large peptides from casein during cheese ripening. Ledford et al. (1966), Reiter et al., (1969) and Green and Foster (1974) also reported that rennet is a major proteolytic factor in Cheddar cheese. However, Green and Foster (1974) and Ohmiya and Sato (1972) suggested that both rennet and microorganisms are responsible for proteolysis in cheese. Rennet was found to accelerate proteolysis by starter bacteria in milk culture (Jespersen, 1966).

Using aseptic vat technique Reiter et al., (1969) showed that cheeses prepared by chemical acidulation instead of using starter culture before renneting had failed to develop flavor whereas cheese made with starter alone did develop flavor. These observations considered in the light of the report by Green (1972) that Cheddar cheese prepared using porcine pepsin (which is completely inactivated during cheese making) is very slow in flavor development lead to the logical conclusion that rennet brings about the majority of casein breakdown to form large peptides. These peptides are broken down to amino acids by microbial enzymes in cheese. Emmons et al., (1960 a,b) showed that some strains of starter organisms produce bitter flavor.

Nonstarter microorganisms also affect cheese flavor. Raw milk with a high bacterial count can give rise to poor-flavored cheese(Smith et al., 1956). Fryer (1969) made a comprehensive review of the role of microflora on cheese ripening.

Relative rates of breakdown of α_s and β casein and the nature of the peptides formed also determine the cheese flavor. Stanley and Emmons (1977) showed that in a normal Cheddar cheese α_s casein was degraded to a greater extent than β casein. The degradation products had higher electrophoretic mobility than the corresponding casein components. Hydrolysis of β casein was implicated in the development of bitter flavor in Cheddar cheese (Phelan et al., 1973). However, bitter peptides may originate from hydrolysis of both α_s and β caseins (pelissier et.al., 1974). Various components in cheeses are in a state of dynamic equilibrium. The large peptides resulting from casein breakdown give rise to amino acids and the latter are then further degraded to amines, aldehydes and ketones, etc. Strecker degradation is an example. In this reaction amino acids and dicarbonyls interact to produce an aldehyde with one less carbon atom than the amino acid (Schonberg and Moubasher, 1952).

$$RCO - CO - R + R'CHNH_2COOH \rightarrow R'CHO + CO_2 + RCHNH_2 - COR$$

All the necessary reactants occur in cheese (Keeney and Day, 1957). These authors also demonstrated the Cheddar flavor in test tube by heating individual amino acid solutions with isatin (Indole-2,3-dione). Derivatives of each amino acid gave rise to a characteristic flavor. Methionine derivative had Cheddar cheese flavor. Strecker degradation products obtained by steam distillation of an incubation mixture of casein solution, proteolytic enzymes, isatin, pyruvic and other keto acids were found to impart Cheddar-like flavor to Cottage cheese curd.

Although protein and carbohydrate derived compounds are necessary for cheese flavor the lipid component and its derivatives are perhaps much more important (Ernstrom, 1974). Ohren and Tuckey (1969)

demonstrated that cheese made of skim milk lacks typical flavor. Free fatty acids formed from amino acids or intermediates of lactose metabolism (Schormüller, 1968) have been reported in all cheese types. Pattern of fatty acid seems to be characteristic of a particular type of cheese. Harper (1959) showed that in Cheddar cheese acetic and butyric acids are the predominant fatty acids whereas propionic acid is the major fatty acid component in a normal Swiss cheese. In Blue cheese fatty acids with 5 and higher number of carbon atoms occur in higher concentration than the short chain fatty acids. Butyric acid is predominant in Provolone cheese. Schormüller (1968) has reviewed the literature on fatty acids of various kinds of cheese.

Kristoffersen et al., (1959) and Bills and Day (1964) determined fatty acids in Cheddar cheese. Neither group found any correlation between free fatty acids and flavor except in cheese with off flavor which had 10 fold increase in free fatty acids. Patton (1964) considered short chain fatty acids to be indispensable for Cheddar cheese aroma.

Carbonyl compounds of various chain lengths have been associated with cheese flavor. Acidic carbonyl compounds reported to be present in cheese are oxalosuccinic, glyoxylic, pyruvic, α-ketoglutaric, acetoacetic and α-ketoisocaproic acids (Bassett and Harper, 1958; Kristoffersen and Gould, 1959; Walker and Harvey, 1959).

Mabbit (1961) reported the following neutral carbonyl compounds in Cheddar cheese: diacetyl, butyraldehyde, acetaldehyde, acetyl methyl carbinol, acetone, methyl ethyl ketone, 2-butanone, 2-pentanone, 2-heptanone, 2-nonanone, 2-undecanone, 2-tridecanone, 3-hydroxybutanone, formaldehyde, 3-methyl butanol, and propionaldehyde. Vedamuthu et al.,

(1966) isolated acetaldehyde, propionaldehyde, acetone, pyruvic acid, diacetyl, glyoxal and an α-ketoalkanal from a milk culture inoculated with a lactic starter known to produce normal flavored Cheddar cheese. A culture that consistently produced fruity flavor in Cheddar cheese was found to produce more carbonyl compounds than the normal starter under the same conditions. Besides it produced formaldehyde instead of propionaldehyde. Beide and Hammond (1979a, b) fractionated Swiss cheese into oil-soluble, water-soluble volatile and water-soluble nonvolatile components. Each of these components were further analysed for individual compounds and their contribution to cheese flavor. Water-soluble volatile fraction was composed of acetic, propionic and butyric acids, diacetyl and ammonia. A synthetic mixture of these compounds reproduced the flavor of water-soluble volatile fraction. The water-soluble nonvolatile fraction contained small peptides and amino acids and these were correlated with the acidity of the fraction, not the lactic acid content or pH. The interaction of calcium and magnesium ions with small peptides and amino acids was attributed to the water-soluble nonvolatile fraction. Free fatty acids were correlated with the nutty flavor of the oil-soluble fraction whereas the neutral portion of the oil-soluble fraction gave undesirable "fermented" flavor. However, the flavor of the whole cheese could not be predicted from those of its individual fractions.

Sulphur compounds have been reported as important in cheese flavor. Singh and Kristoffersen (1970) accelerated flavor development in Cheddar slurry by treatment with reduced glutathione. Singh and Kristoffersen (1971) also made the same observation about Swiss cheese flavor. Manning (1978) correlated Cheddar cheese flavor with

the concentrations of H_2S and methanethiol. He concluded that reduced sulphur compounds, e.g. DTT, GSH which are known to accelerate flavor development do so by providing necessary redox potential to form methanethiol from its precursor, namely methionine. Degradation of sulphur-containing amino acids by Strecker degradation has also been considered as important for flavor.

From above discussion it seems that our understanding of cheese flavor is far from clear. Monitoring one or more of the flavor associated compounds gives an indication of the rate and stage of the ripening process but not a complete picture of the cheese quality. "Component balance theory" of Kosikowski and Mocquot (1958) seems to explain flavor development more logically. According to this theory only a small number of compounds are responsible for cheese and other food flavors. Some of these compounds are common to all cheeses and are responsible for background flavor. Flavor of a particular cheese type results from blending of a number of these compounds in a definite proportion with respect to the background. Intensification of a normal flavor results when concentrations of all these compounds increase, maintaining the balance. For example, in a good Cheddar cheese the amino acids, peptides, carbonyls, fatty acids, salts and residual para-casein may be present in certain proportion to impart background flavor and characteristic Cheddar flavor may be perceived when the volatile sulphur compounds (Manning, 1978) are present in the cheese in a definite proportion with respect to each other and also with respect to the background.

Like flavor, texture is also an important characteristic of cheeses, and the biochemical changes occurring during ripening also

influence the texture of cheeses as well. Milk-coagulating enzymes contribute to cheese texture either by retention of fat during curdling and also through proteolysis (Stanley and Emmons, 1977; Stanley et al., 1980).

MATERIALS AND METHODS

I. Seal Stomachs

Stomachs from harp seals (Pagophilus groenlandicus) were collected by officials of the Department of Fisheries and Oceans, St. John's in 1979 and 1980. The seals were caught off the coast of Newfoundland during the hunting season in March. The stomachs collected in 1979 were from approximately two-week-old pups. These stomachs were shipped frozen. On arrival in St. John's the stomachs were cleaned and freeze-dried.

The stomachs collected in 1980 were from both 2-week-old and 2-year-old seals, shipped fresh chilled on ice. On arrival the stomachs were cleaned, the mucosal layer separated and freeze-dried. The wet weights of the clean stomachs from two-week-old pups were from 30 to 75 grams and those from the two-year-old seals were from 180 to 250 grams. The freeze-dried samples were stored at -20°C until used.

II. Chemicals

N-acetyl-L-phenylalanyl-L-diiodotyrosine (APDT), ninhydrin, substrate grade hemoglobin, yeast ribonucleic acid (RNA), disodium ethylenediamine-tetraacetic acid (EDTA), acrylamide, N,N'-methylene-bis-acrylamide (BIS), N,N,N',N'-tetramethylethylenediamine (TEMED), ammonium persulphate, riboflavin, penicillin, streptomycin, bovine pancreatic ribonuclease (RNAase E.C.2.7.7.16), twice-crystallized porcine pepsin (E.C.3.4.4.23.1), and calf chymosin (E.C.3.4.23.4) were purchased from Sigma Chemical Co., St. Louis, MO. Diethylaminoethyl

Sephadex A-50 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Carbobenzoxy-D-phenylalanyl-triethylene-tetramine-Sepharose (Z-D-Phe-T-Sepharose) was purchased from Pierce Chemicals, Ill. Hansen's buttermilk culture and calf rennet were purchased from Horan Lally Co., Mississauga, Ontario. Mucor miehei protease (Fromase 100) was a gift from G.B. Fermentation Industries Inc., Ill.

All other chemicals were of the highest purity available and purchased from various commercial sources.

III. Preparation of crude enzyme

Enzyme used in preliminary studies discussed in the introduction was prepared following the method of Qadri et al., (1962) with a slight modification. Freeze-dried whole stomach (see Section I) was pulverised in a Waring blender at the maximum setting in the cold room at 4°C for a total of 2 minutes with 30-second bursts and a cooling period of 2 minutes in between. The powder was stored at -20°C until used.

For extraction of enzymes 1 gram of the powder was stirred with 20 ml of 10% acetic acid at 4°C for 12 h. The homogenate thus obtained was adjusted to pH 5.3 by adding 1M NaOH and centrifuged in a Sorval RC-5 superspeed refrigerated centrifuge for 30 minutes at 38,000xg. The supernatant was concentrated about 10 fold by ultrafiltration in a 43 mm Amicon ultrafiltration apparatus fitted with a PM_{10} membrane using nitrogen gas as a propellant. This preparation was used in the preliminary work discussed in the introduction (results in Appendices A-E).

IV. Isolation and Purification of zymogens

(a) Extraction of crude zymogens: The freeze-dried samples of gastric mucosa from two week old and two year old seals collected in 1980 (see Section 1) were pulverised as in Section III. One gram of pulverised mucosa was suspended in 20 ml of buffer A (20 mM sodium phosphate pH 7.2 containing 5000 units of penicillin, 50 mg and streptomycin per litre) and stirred gently at 4°C for 12 h. The slurry thus obtained was centrifuged at 48,000xg at 4°C for 30 minutes. The pellet was resuspended in 10 ml of buffer A and centrifuged again. The two supernatants were combined, and centrifuged at 134,000xg for 60 minutes at 4°C in a Beckman L3-50 ultracentrifuge. The supernatant was used as a crude preparation of zymogens. This was converted to enzyme by adjusting the pH to 2.0 with 1M HCl and incubating at 30°C for 30 minutes when needed.

(b) Ion-exchange chromatography: Diethylaminoethyl Sephadex A-50 (DEAE-Sephadex A-50) was packed into a 2.5 x 30 cm column following the manufacturer's instruction. The column was equilibrated with buffer A for 24 h before use.

Forty ml of crude zymogen preparation obtained as described above (IVa) were dialysed against 2x2L buffer A at 4°C and applied to the ion exchange column. Five ml fractions were eluted with buffer A until the absorbance at 280nm of the fractions and their proteolytic activity approached zero. The column was then eluted by stepwise addition of 0.1M, 0.2M and 0.8M NaCl to buffer A.

(c) Gel filtration: An 88 x 1.6 cm column was packed with Sephadex

G-100 according to manufacturer's instruction and washed with buffer B (buffer A containing 1 mM dithiothreitol, 1 mM disodium ethylene-diamine tetraacetic acid, and 0.5M NaCl).

Fractions eluted from the DEAE-Sephadex column with potential proteolytic activity were pooled, concentrated by ultrafiltration and 4 ml of the concentrate were applied through the bottom of the column and eluted with buffer B in the ascending mode. The column was previously calibrated with a mixture of standard molecular-weight markers consisting of bovine serum albumin (65,000 daltons), chicken egg albumin (43,000 daltons), sheep prolactin (23,800 daltons), cytochrome C (12,700 daltons), and blue dextran (2,000,000 daltons).

(d) Affinity-column chromatography: The method of Fujiwara and Tsuru (1977) for purification of pepsin was followed with necessary modifications for zymogen. Ten ml of carbobenzoxy-D-phenylalanyl-triethylene-tetramine-sepharose (Z-D-Phe-T-sepharose) were packed into a 10-ml disposable plastic syringe with glass wool to support the gel. Two ml of concentrated zymogen A from DEAE-Sephadex column were mixed with 4 ml of the starting buffer and applied to the column and eluted quickly with the same buffer (30 mM acetate pH 3.5, containing 0.1M NaCl). When the absorbance of the fractions at 280 nm reached the baseline the column was washed with 1M sodium acetate p^H 3.5 containing 1M NaCl. The adsorbed zymogen was then eluted with the starting buffer containing 1M guanidine chloride. Aliquots from fractions were incubated with hemoglobin to monitor activity and the fractions eluted with the buffer containing guanidine chloride were pooled and mixed with an equal volume of 0.2M sodium phosphate buffer

pH 7.2. The mixture was then dialysed against 2L of water at 4°C for 3 h and concentrated by ultrafiltration using a PM10 membrane.

V. Determination of Enzyme Activity

(a) Measurement of pepsin activity: The method of Bohak(1970) was followed to determine the proteolytic activity of enzymes using hemoglobin as the substrate with the following modification. A 7.5% (w/v) solution of bovine hemoglobin in 0.06N HCl was dialysed against 50 volumes of 0.06N HCl at 4°C with 2 changes of the acid at 4 h intervals. The dialysed solution was then freeze-dried and stored in screw cap bottles at 4°C until used. Dialysis removed most of the trichloroacetic acid (TCA)-soluble materials that absorbed at 280 nm.

For assay, 8 ml volume of a 2% hemoglobin solution in 0.06N HCl (pH 1.8) was equilibrated at 30°C in a water bath and incubated with 1.0 ml of proteolytic enzyme. At various time intervals, 0.8-ml aliquots were removed, and the reaction stopped by mixing with 1.0 ml of 6% TCA. After cooling on ice for 30 minutes the samples were centrifuged at approximately 3,000xg for 30 minutes. The absorbance of the supernatant at 280 nm was measured in a Beckman DU 8 spectrophotometer. For control 0.8 ml solution of hemoglobin was incubated in duplicate for the same length of time and the enzyme added after TCA. One pepsin unit is the amount of enzyme that produces an absorbance increase of 0.0084 per minute under this condition. Enzyme activity measured with hemoglobin as the substrate has been termed pepsin activity or proteolytic activity in this study.

(b) Milk-clotting assay: The milk clotting activity of proteases was

determined following the method of Berridge (1945) except that the volumes of the Berridge substrate (12 g skim milk powder suspended in 100 ml 0.01M $CaCl_2$) and enzyme were scaled down to 1.0 ml and 0.1 ml respectively. Instead of clotting milk sample in 100 seconds the concentration of the enzyme was adjusted to clot milk in 600 $\pm$ 10 seconds in order to minimize personal error. Milk-clotting unit used in this study was as defined by Berridge (1945). One clotting unit is the amount of enzyme that clots 10 ml of milk in 100 seconds.

(c) APDT assay: Method of Ryle (1970) was followed to determine the rate of enzymatic hydrolysis of N-acetyl-L-phenylalanyl-L-diiodo-tyrosine (APDT), except for volumes of substrate and enzyme. To 2.45 ml of 0.01N HCl was added 0.7 ml solution of 0.002M APDT in 0.005M NaOH. After equilibration to 30°C, 0.35 ml of enzyme was added. At intervals 0.4 ml of sample was pipetted out, mixed with 0.2 ml each of the cyanide acetate buffer and ninhydrin solution. After heating for 15 minutes on a boiling water bath, the samples were cooled, and 1 ml of 50% isopropanol solution was added and vigorously shaken. The absorbance of the colour so developed was recorded at 570 nm. The blank incubation mixture contained 0.005M NaOH instead of APDT.

(d) Inactivation of ribonuclease: The method of Bang-Jensen et al., 1964 was used for the enzymatic degradation of ribonuclease. Ribonuclease was dissolved in 0.05M citrate/HCl buffer, pH 2.6 prior to incubation with crude seal gastric proteases and the isoenzymes A and B. For porcine pepsin and seal protease C, ribonuclease was dissolved in

0.01M HCl and for calf chymosin it was dissolved in 0.05M citrate/HCl pH 3.5. The incubation of ribonuclease with proteases was carried out for 2 h instead of 40 minutes and the digestion stopped by adding 0.25 ml of 1.5M sodium phosphate pH 7.0.. The residual ribonuclease was diluted by adding 14 ml of water. After 5 minutes 0.1 ml of this diluted mixture containing the residual ribonuclease was added to a reaction mixture consisting of 0.65 ml of 0.1M sodium acetate (pH 5.0), 0.5 ml of 1% RNA in the same buffer. Incubation was carried out at 25°C. After 24 minutes 0.25 ml of 0.75% uranyl acetate in 24% perchloric acid was added to stop the reaction. After cooling on ice for 30 minutes, the samples were centrifuged at 3000xg for 30 minutes at room temperature. Thirty µl of the supernatant were added to 1.0 ml water in a 1 cm quartz cuvette. After thorough mixing the absorbance at 260 nm was noted. To determine any possible ribonuclease in the protease samples the latter were incubated with respective buffers instead of ribonuclease. To determine A260nm-absorbing materials in RNA which are soluble in uranyl acetate, a blank reaction was set up with 0.75 ml of sodium acetate and 0.5 ml of 1% RNA. After incubation at 25°C for 24 minutes 0.25 ml of uranyl acetate was added and the absorbance at 260 nm of the supernatant was determined as described above.

(e) Determination of the denaturation of proteases by urea: One volume of the respective protease, dissolved in 0.01M HCl was incubated with two volumes of 9M urea in 0.15M sodium acetate, pH 5.64. The final pH of the mixture was 5.40. At intervals 30 µl aliquots were withdrawn in duplicate and added to 0.8 ml of 2% hemoglobin in 0.06M

HCl. After 30 minutes of incubation at 30°C the reaction was stopped by adding 1.0 ml 6% TCA. The absorbance of the TCA soluble product at 280 nm was determined as described in section V(a). The stock enzymes were standardized so that a 30 µl aliquot of the urea-acetate-enzyme mixture would produce an initial increase in A280 nm of ca 0.5 in 30 minutes under the assay conditions.

VI. Determination of the pH optima for pepsin activity

A 2% solution of hemoglobin in citrate buffer pH 1.2 was used to titrate 5 ml volumes of another 2% hemoglobin solution in sodium acetate buffer pH 5.0 to make hemoglobin solutions of various pH values between pH 1.2 and 5.0. Both the buffers used in making the stock solutions had the same ionic strength (μ=0.05). A 0.8 ml volume of each hemoglobin solution was then incubated with 30 µl of enzyme containing 0.4 pepsin unit at 30°C for 60 minutes, and the reaction stopped by adding 1.0 ml of 6% TCA. The absorbances at 280 nm of the supernatants were determined as described in Section V(a).

VII. Determination of the influence of pH on the stability of the proteases.

Five hundred microlitres of 10 mM sodium acetate pH 5.3 containing 24-28 pepsin units of the respective proteases were mixed with an equal volume of a series of buffers of ionic strength 0.1. One drop of toluene was added to each of the mixtures, the tubes were stoppered and incubated at 25°C. After 24 h 50 µl aliquots of the mixtures were assayed for residual pepsin activity as described above (Section V(a)) except that the 2% hemoglobin solution was prepared in 0.2M

citrate-HCl buffer pH 1.8.

VIII. Isoelectric focusing

Preparative isoelectric focusing of the crude activated enzyme on Sephadex IEF was done on a Pharmacia flat-bed isoelectric focusing apparatus following the manufacturer's instruction. Pharmalyte 3-10 was used as pH gradient. The gel was prefocused for 30 minutes at 8-watt setting of the Pharmacia electrophoresis constant power supply model ECPS 3000/150. After applying the sample, the gel was focused for 9 h at 30-watt setting. The gel was cooled by circulating water maintained at 4-8°C. The gel was then divided into 21 equal sections, scooped off the flat-bed into test tubes, and the pH of the fractions was measured. The gel fractions were then placed in a hypodermic syringe plugged with glass wool. The protein was then eluted from the gel. Fifty microliter aliquots were then used to detect the proteolytic activity by incubating with 0.8 ml hemoglobin solution for 60 min. Fractions with proteolytic activity were pooled into 4 portions to determine CU:PU.

Analytical polyacrylamide gel isoelectric focusing of purified zymogens A and C was carried out using Pharmalyte 3-10 as the pH gradient. The gel was cast in a 115 x 230 mm glass plate following the manufacturer's suggestion. Samples (15-20 µl) were applied directly on the surface of the gel. The focusing was carried out for 1 h at 3000V and 20W setting using the power supply described above. After focusing, the pH of the gel was measured using a surface electrode. The protein was stained and destained by following the manufacturer's instruction.

IX. Electrophoresis

Polyacrylamide gel electrophoresis at pH 8.3 was performed following the method of Davis (1964). Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis in 7.5% separating gel and 3% stacking gel was done according to Laemli (1970). Unless otherwise indicated, the gels were cut off at the marker dye after electrophoresis. The gels were stained for 30 minutes in 0.1% Coomassie blue in 50% methanol, and 10% acetic acid and destained by diffusion in a solution of 5% methanol and 10% acetic acid. Bovine serum albumin (MW 65,000 daltons), chicken egg albumin (MW 43,000 daltons), sheep prolactin (MW 23,800 daltons) and cytochrome C (MW 12,700 daltons) were used as standard molecular weight markers.

X. Amino acid analysis

Amino acid analysis was performed in a Beckman Model 121MB amino acid analyser using the method described in the Beckman bulletin 121M-TB-013.

XI. Determination of nitrogen and protein estimation

Total and nonprotein nitrogen in whey were determined following the method of Rowland (1938). Total and nonprotein nitrogen products of enzymatic casein digestion were determined according to the method of Lang (1958).

Protein estimation was carried out following the method of Hartree (1972).

XII. Preparation of Acid Casein

Pasteurised skim milk (Sunshine Dairy, St. John's, Nfld.) was used for the isolation of acid casein at pH 4.6 following the method of Fox and Guiney (1972). The product was freeze-dried and stored at 4°C until used.

XIII. Cheddar Cheese

(a) Preparation: Cheddar cheese was prepared using pasteurised whole milk (Sunshine Dairy, St. John's, Nfld.) by following the method of Kosikowski (1978), adapted for a small volume of milk. Eighteen liters of milk were used for each of the coagulants - calf rennet, activated DEAE peak A, crude SGP, and Mucor miehei protease (Fromase 100). DEAE peak A material and crude zymogen from two week old seal were dialysed against 2x2L of 20 mM sodium phosphate pH 7.2 before activation at pH 2.0, in order to remove penicillin and streptomycin used during the extraction of the enzymes (Materials and Methods, Section IV(a)).

The cheese from an 18L batch was pressed in one lot using a press constructed with a plastic barrel, 7 inches in diameter following the method of Radke (1974). After pressing for 18 h in this barrel the cheese block was split into 4 smaller blocks and pressed in perforated square plastic boxes (12 x 12 cm and 5 cm high) lined with cheesecloth. Three bottles each containing 16 gallons of water were used to press these blocks for another 8 h. The cheese blocks (slightly more than a pound each) were vacuum-packed and cured at 8°C in a Hotpack temperature-humidity chamber (Hotpack Canada Ltd., Ontario).

(b) Chemical analyses: Protein, fat and moisture in cheese were determined following the methods of A.O.A.C. (1970). pH of cheese was determined by mixing 5 g of cheese with 5 ml of deionised water using a glass electrode.

(c) Sensory evaluation of cheese: Cheeses were evaluated by preference test (Larmond, 1977) at the taste panel room in the Department of Fisheries and Oceans, St. John's. Thirty untrained panelists were provided with number-coded cheese samples (approx. 3x3x2 cm), and general information about Cheddar cheese. Panelists were asked to rate the samples as to overall preference on a numerical scale ranging from 9 (like extremely) to 1 (dislike extremely). The data were evaluated by analysis of variance (Larmond, 1977).

RESULTS AND DISCUSSION

I. Extraction of crude enzyme

Crude seal gastric protease (SGP) used in the preliminary studies (See Appendices A-E) was prepared following the method of Qadri et al., (1962) modified as described in Materials and Methods (Section III). Approximately 4.5 clotting units were obtained per gram of whole stomach powder from a 2-week-old seal pup collected in 1979 (Section I, Materials and Methods). It was later found that extraction with 20 mM phosphate pH 7.2 (buffer A, Materials and Methods, Section III) for 12 h yielded 26.3 clotting units per gram of the same stomach powder. The latter method extracts the zymogens while the method of Qadri et al., (1962) extracts the activated gastric proteases. The lower yield obtained following the method of Qadri et.al., may be attributed to autodigestion of the proteases. Based on this observation, phosphate buffer was used for extraction in subsequent studies. Also, instead of the whole stomach the mucosal layer was used in order to have higher specific activity of the starting materials. The stomachs used for this purpose were from both two-week-(approximately) and two-year-old (approximately) harp seals collected in 1980.

The average yield of activated zymogen extracted from mucosal powder was 191.5 clotting units/g for two-week-old pups and 262.5 clotting units/g for two-year-old seals. These averages are based on measurements on two stomachs from adults and three stomachs from pups. The yield of zymogen from whole stomach collected in 1979 was much less (26.3 clotting units/g whole stomach powder) than from the

stomachs collected in 1980, because (1) only the mucosa was extracted from the stomachs collected in 1980 whereas in the case of the stomachs collected in 1979 the freeze-dried powder of whole stomach was extracted, and (2) the 1980 stomachs were empty but the 1979 stomach was full of curdled milk, indicating that some of the zymogens had already been secreted. The fact that the 1980 stomachs were shipped chilled on ice while the 1979 stomach was shipped frozen and the possible difference between individuals of the same species may also have contributed to the differences observed.

II. Proteolytic activity

Hemoglobin solution (2% in 0.06N HCl, pH 1.8) was used routinely as substrate to monitor the proteolytic activity of the enzymes at various stages of purification. Zymogens were activated by incubating at pH 2.0 for 30 minutes at 25°C (Results and Discussion, Section V). However, this preactivation step was omitted when aliquots of individual fractions from chromatographic column eluates were assayed to monitor their proteolytic activities. The rate of hemoglobin hydrolysis at pH 1.8 and 30°C was linear with time up to 120 minutes when the enzyme concentration was 1.9 pepsin units per ml of incubation mixture (Figure 1). In a 30 minute incubation under this condition the rate of hydrolysis was also linear as a function of the concentration of the crude enzyme (Figure 1b).

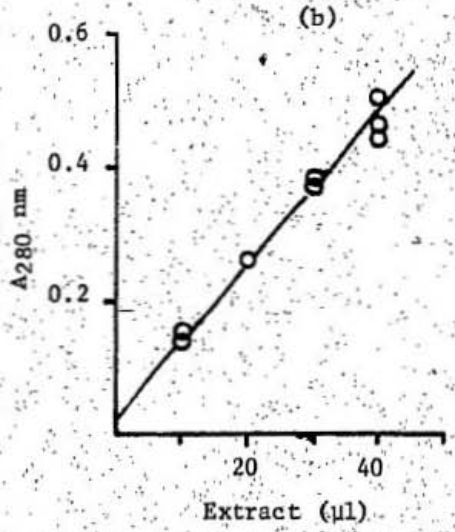
III. Ion exchange chromatography

As shown in Figures 2a and 2b, crude preparations of zymogens from the mucosa of young and adult seal stomachs separated into 4

Figure 1. Proteolytic activity as functions of time and enzyme concentration.

(a) A 2% hemoglobin solution in 0.06M HCl was incubated with 1.9 pepsin units of crude extract per ml of reaction mixture at 30°C. At intervals indicated duplicate samples of 0.9 ml were withdrawn and the proteolytic activity determined as described in Materials and Methods.

(b) A 2% hemoglobin solution in 0.06M HCl was incubated with the indicated volumes of a dilute solution of crude enzyme so that the final volume of reaction mixture was 0.9 ml. After 30 minutes of incubation at 30°C, the raction was stopped by adding TCA as described in Materials and Methods and extent of proteolysis determined. Absorbances due to control were subtracted for each enzyme concentration.


(b)
0.6
0.4
0.2
A280 nm
20
40
Extract (µl)

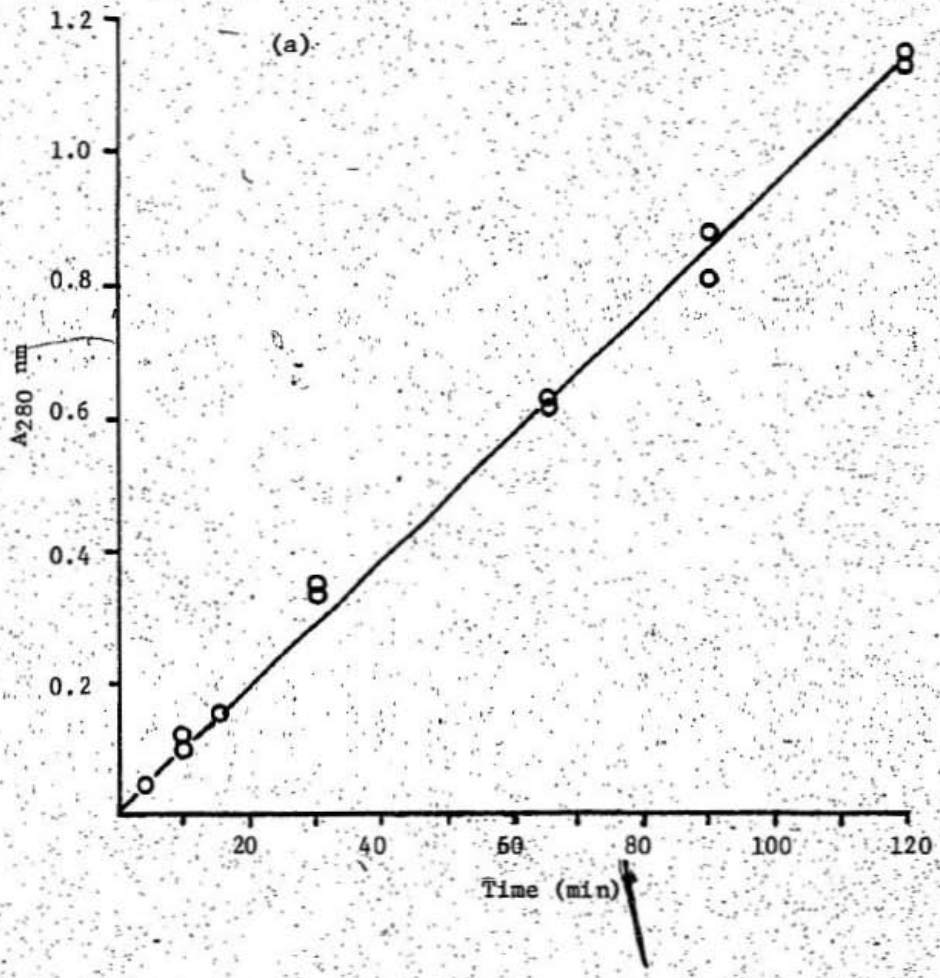
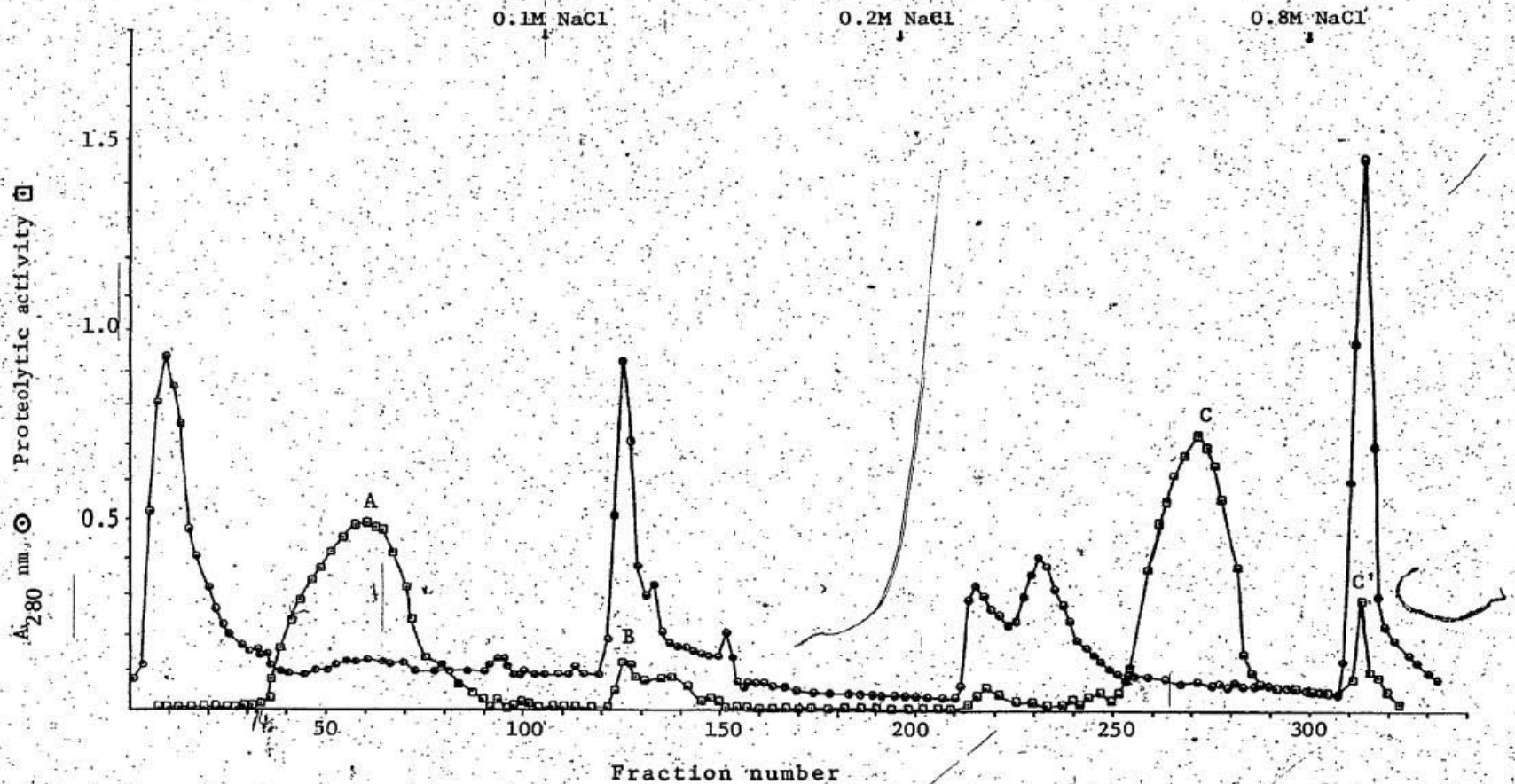

(a)
1.2
1.0
0.8
0.6
0.4
0.2
A280 nm
20
40
60
80
100
120
Time (min)

Figure 2. Ion exchange chromatography of extracts from gastric mucosa of harp seal.

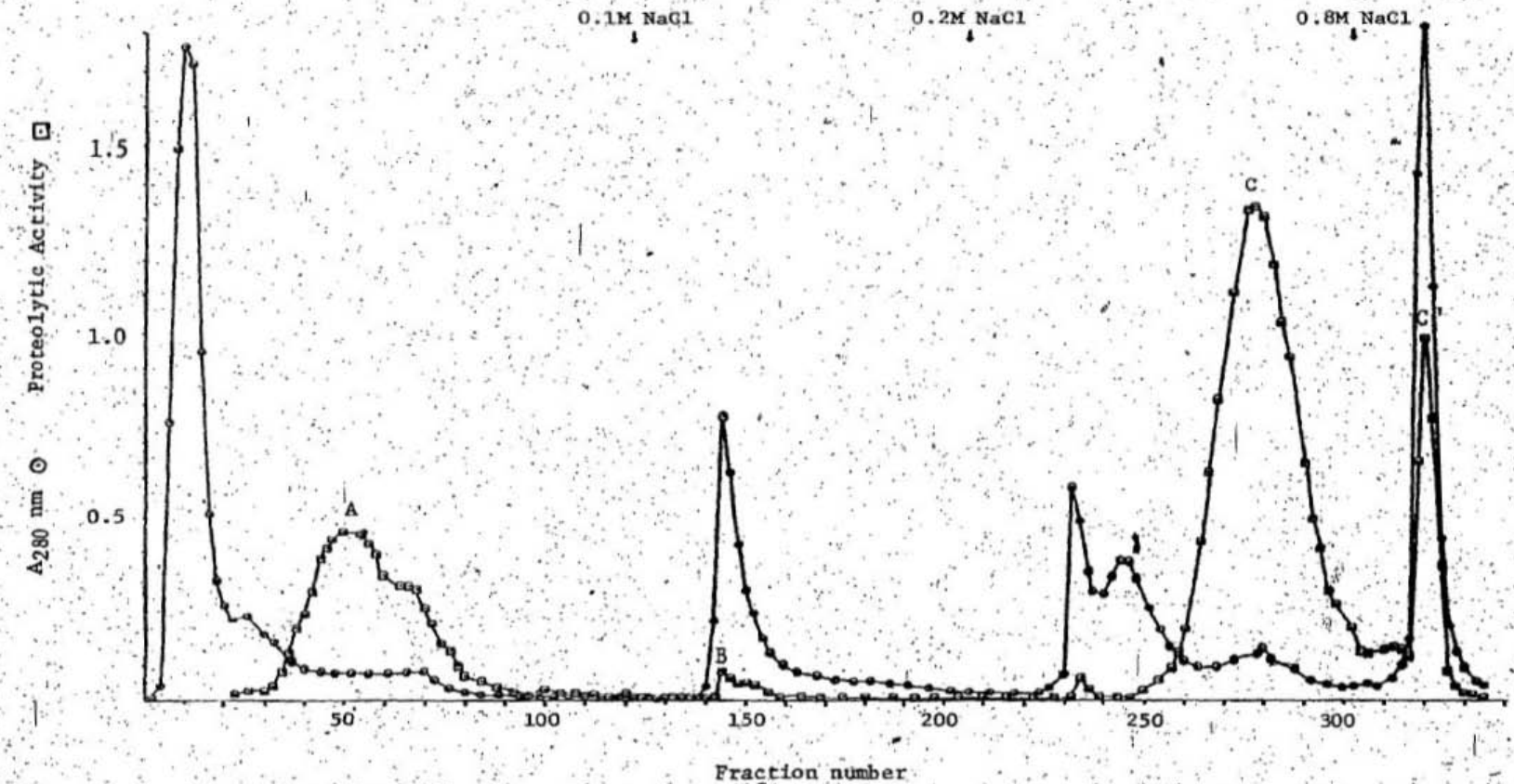
Forty ml of homogenate from 2g of dry mucosa were applied to a 2.5x 30 cm DEAE Sephadex A-50 and eluted with buffer A untill the A280nm and the proteolytic activity approached zero. The column was then eluted stepwise addition of NaCl to buffer A as indicated. Twenty ul aliquots from alternate fractions were incubated with hemoglobin at 30°C for 3h to monitor potential proteolytic activity. Flow rate 20 ml/h, fraction size 5.0 ml. (a) homogenate from a 2-week-old seal, (b) homogenate from a 2-year-old seal. The zymogens are activated in the test procedure for proteolytic activity at p^H 1.8.

⊙, Absorbance at 280nm; ⊡, enzyme activity, absorbance of TCA soluble fraction at 280nm.

(a)


0.1M NaCl
0.2M NaCl
0.8M NaCl
1.5
1.0
0.5
A
B
C
C'
50
100
150
200
250
300
Fraction number
Proteolytic activity
$A_{280\ nm}$

(b)


0.1M NaCl
0.2M NaCl
0.8M NaCl
A280 nm ⊙ Proteolytic Activity ⊡
1.5
1.0
0.5
A
B
C
C'
50
100
150
200
250
300
Fraction number

pepsinogen components, A, B, C and C' of which B and C' were minor and of low specific activity. No further purification of the components B and C' was attempted. The relative proportion of zymogen A as pepsin activity from stomachs of two-week-old pups was higher than that from adult stomach. As shown in Table 1 the activity of peak A was 44.5% of the total recovered from one stomach and 51% in the other. The proportion of peak C was 48.5% and 37.8%, respectively. These estimations were based on the areas of the peaks in the activity profiles shown in Figures 2a and 2b. In the adult-stomach mucosa peak A activity represented 32.5% in one run and 27% in the other. The proportions of peak C activity in those two samples were 54.8% and 59.1%, respectively. These two samples were from the same animal. Compared with peaks A and C, the yields of peaks B and C' were highly variable in both the young and the adult-mucosal samples.

Multiple isoenzyme forms of both chymosin and pepsin have been reported in other species as well. Occurrence of 4 calf chymosins from their corresponding zymogens has been reported by Asato and Rand (1977). Amer et al., (1980) separated "adult bovine rennet" into two components. The major component was pepsin-like with a CU:PU ratio of 0.032 and the minor component chymosin-like with a CU:PU ratio of 0.829. However, the authors did not define the units of milk-clotting and proteolytic activities. Foltmann et al., (1978) showed that the ratio of milk clotting activity to general proteolytic activity of piglet chymosin was 30 and 70 times as high as those for pepsins from adult fundus and pylorous respectively and 4 times as much as that for calf chymosin.

Table 1. Relative proportions of various DEAE peaks from mucosal homogenates of the stomachs of young and adult harp seals

Age	CU[1]/PU[2] of Crude homogenate	Relative Proportions[3] of Peak %			
		A	B	C	C'
2 week[4]	0.024	44.5	4.1	48.5	2.9
	0.028	51.0	5.0	37.8	6.2
2 years[5]	0.018	32.5	3.7	54.8	9.0
	0.016	27.0	1.8	59.1	2.1

Notes:
1. One C.U. (clotting unit) is the amount of enzyme that will clot 10 ml of 12% skim milk in 100 seconds.
2. One pepsin unit (PU) is the amount of enzyme that produces an increase in the absorbance at 280 nm of TCA-soluble hydrolysis products of 0.0084 per minute under the conditions described in text.
3. Based on pepsin activity (see fig. 2a, b).
4. Stomachs from two different animals.
5. Samples from the same stomach.

IV. Purifications of zymogens A and C

(a). Gel filtration: The two major zymogens A and C were purified by molecular-sieve chromatography using Sephadex G-100 as described in Materials and Methods (Section IV(c)). Figure 3 shows the protein profile and the pepsin activities of the chromatographic fractions. The column was calibrated with standard molecular weight markers. The peak peptic activity was eluted in a position corresponding to a molecular weight of 26,300 ± 1540 daltons for zymogen A based on 5 trials. The molecular weight of zymogen C was estimated at 37,000 ± 1075 daltons from two trials.

(b). Purification of zymogen A by affinity chromatography: The zymogen representing peak A on the DEAE-Sephadex column was also purified by chromatography with carbobenzoxy-D-phenylalanyl-triethylene-tetramine-Sepharose (Z-D-phe-T-Sepharose), as described in Materials and Methods (Section IV(d)). The major portion of the protein applied to the column was eluted unadsorbed and had very little proteolytic activity (Figure 4). Most of the proteolytic activity was eluted with the buffer containing guanidine-HCl. The active fractions were pooled, mixed with equal volume of 0.2M sodium phosphate (pH 7.2) in order to prevent the conversion of the zymogen to the active enzyme. This mixture was then dialysed against water at 4°C. The dialysate was then concentrated by ultrafiltration to 1.9 ml. The preparation so obtained had some of the zymogen activated as evident from a milk-clotting activity of 2.0 clotting units/mg of protein. After activation at pH 2.0 the specific activity increased to 39.4 clotting units/mg (Table 2).

Figure 3. Chromatography of DEAE peaks on Sephadex G-100 column.

Fractions from DEAE-Sephadex A-50 column with proteolytic activity were pooled, concentrated and 2 ml of the concentrated material were applied to a Sephadex G-100 column (88 x 1.6 cm) equilibrated with buffer B. The column was eluted with the same buffer in the ascending mode. Fraction size 2.7 ml, flow rate 5.0 ml/h. Fifty µl aliquots from alternate fractions were used to monitor potential proteolytic activity. ○, A_{280} of fractions; □, Proteolytic activity, optical density change at 280nm.

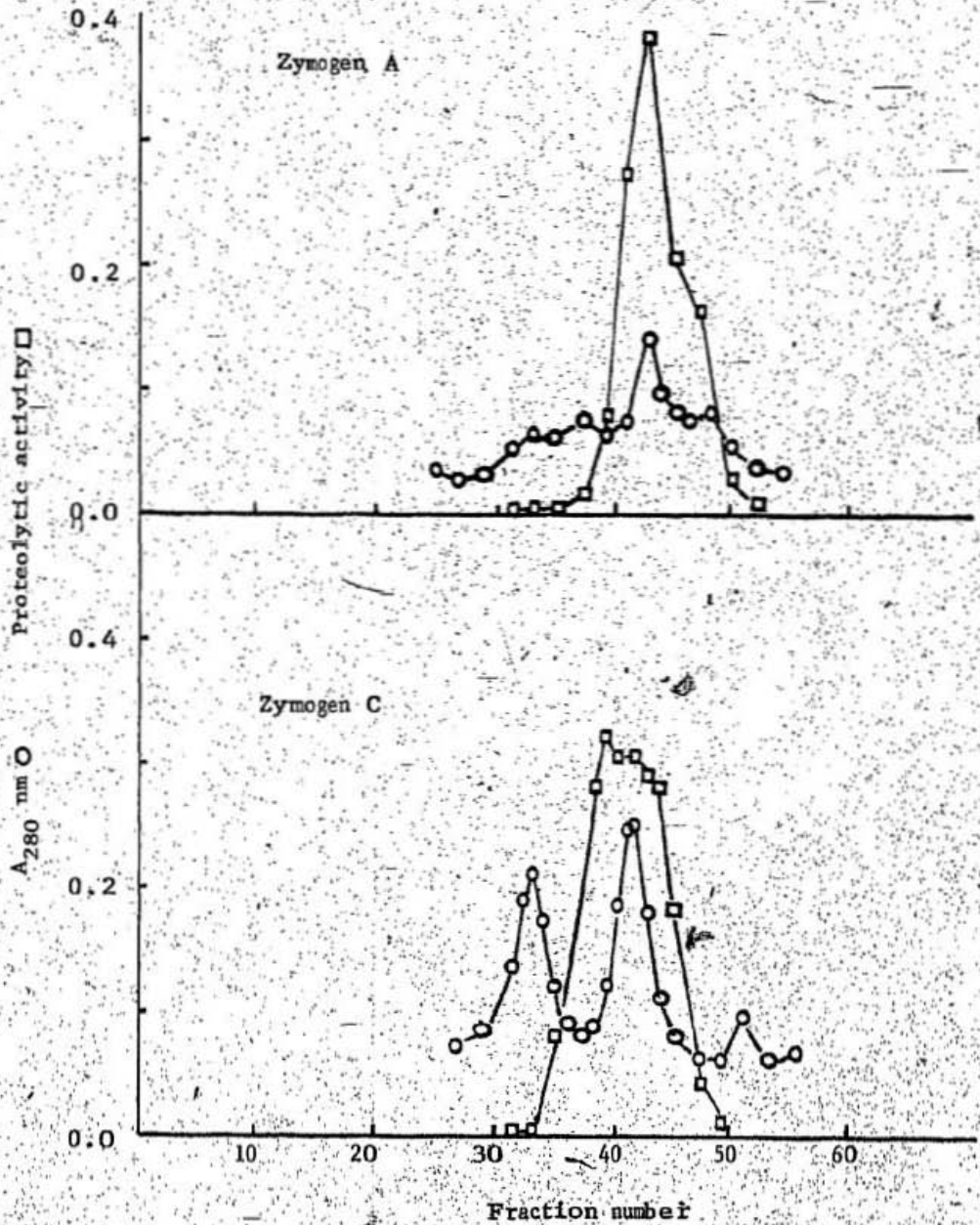

Zymogen A
Zymogen C
Proteolytic activity
A_{280} nm
0.4
0.2
0.0
0.4
0.2
0.0
10
20
30
40
50
60
Fraction number

Figure 4. Affinity chromatography of Zymogen A on CBZ-D-phenylalanyl-TETA- Sepharose 4B.

Two ml of concentrated material from DEAE peak A were chromatographed on a column packed with carbobenzyxy-D-phenylalanyl-triethylene-tetramine Sepharose (Z-D-Phe-T-Sepharose) as described in Materials and Methods. Buffer changes are indicated by arrow. Twenty µl aliquots from alternate fractions were incubated with hemoglobin solution for 60 minutes to monitor potential proteolytic activity. Flow rate 50 ml/h; fraction sizes 2.5 ml. ■, Absorbance at 280nm of fractions, ●, proteolytic activity, change in absorbance at 280nm.

Figure 5. Time-course of activation of zymogens.

To 1.0 ml of buffer A containing Zymogen A, B, or C, 24 μl of 1M HCl were added to adjust the pH to 2.0 at 25°C. Immediately after adding HCl and at intervals indicated 20μl aliquots were removed in duplicate to monitor milk-clotting activity. ▲, Zymogen A; ●, Zymogen B, and Δ, Zymogen C.

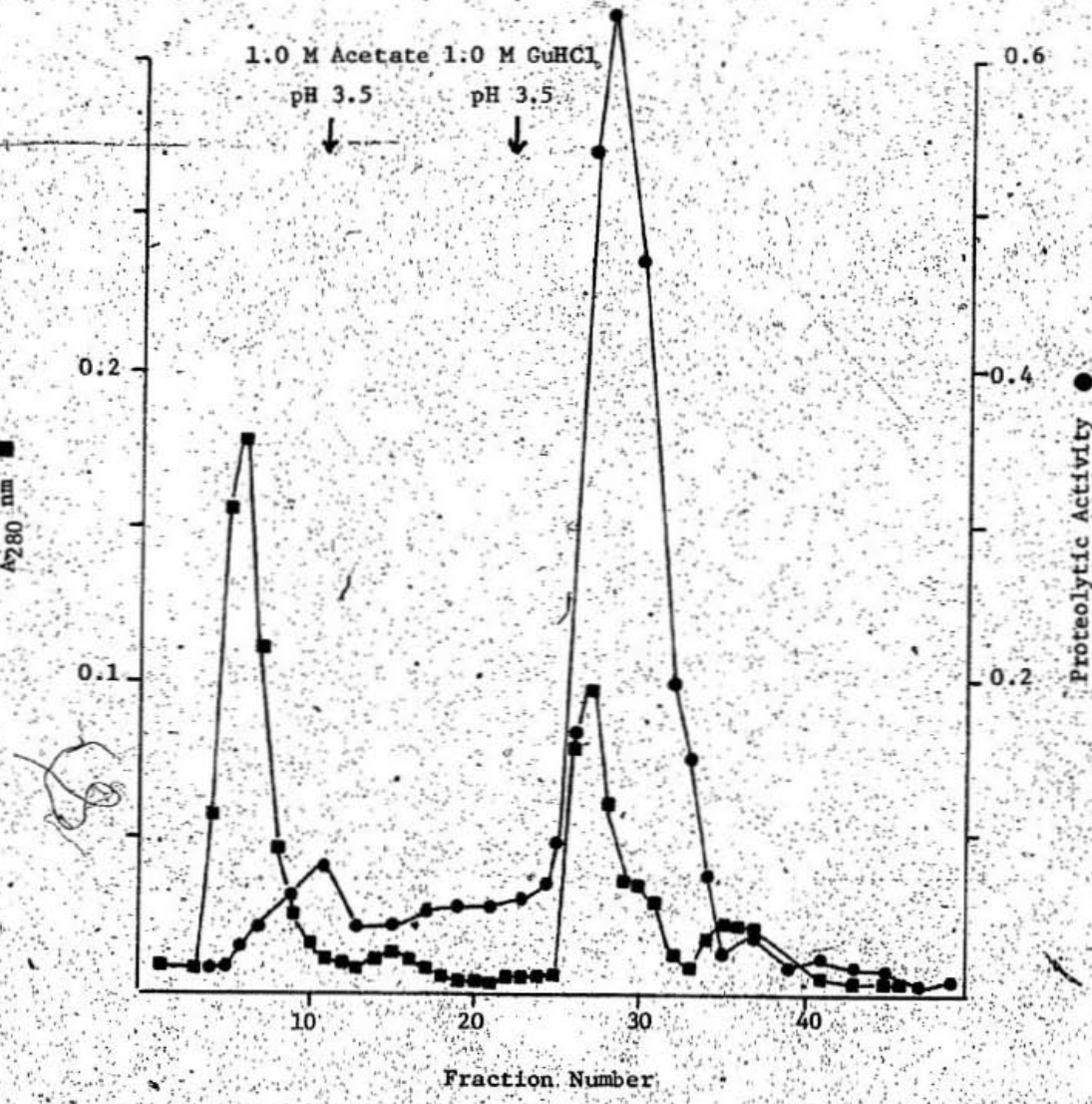
Table 2. Purification of Zymogens A and C from Mucosal Homogenate of a 2 year old seal stomach

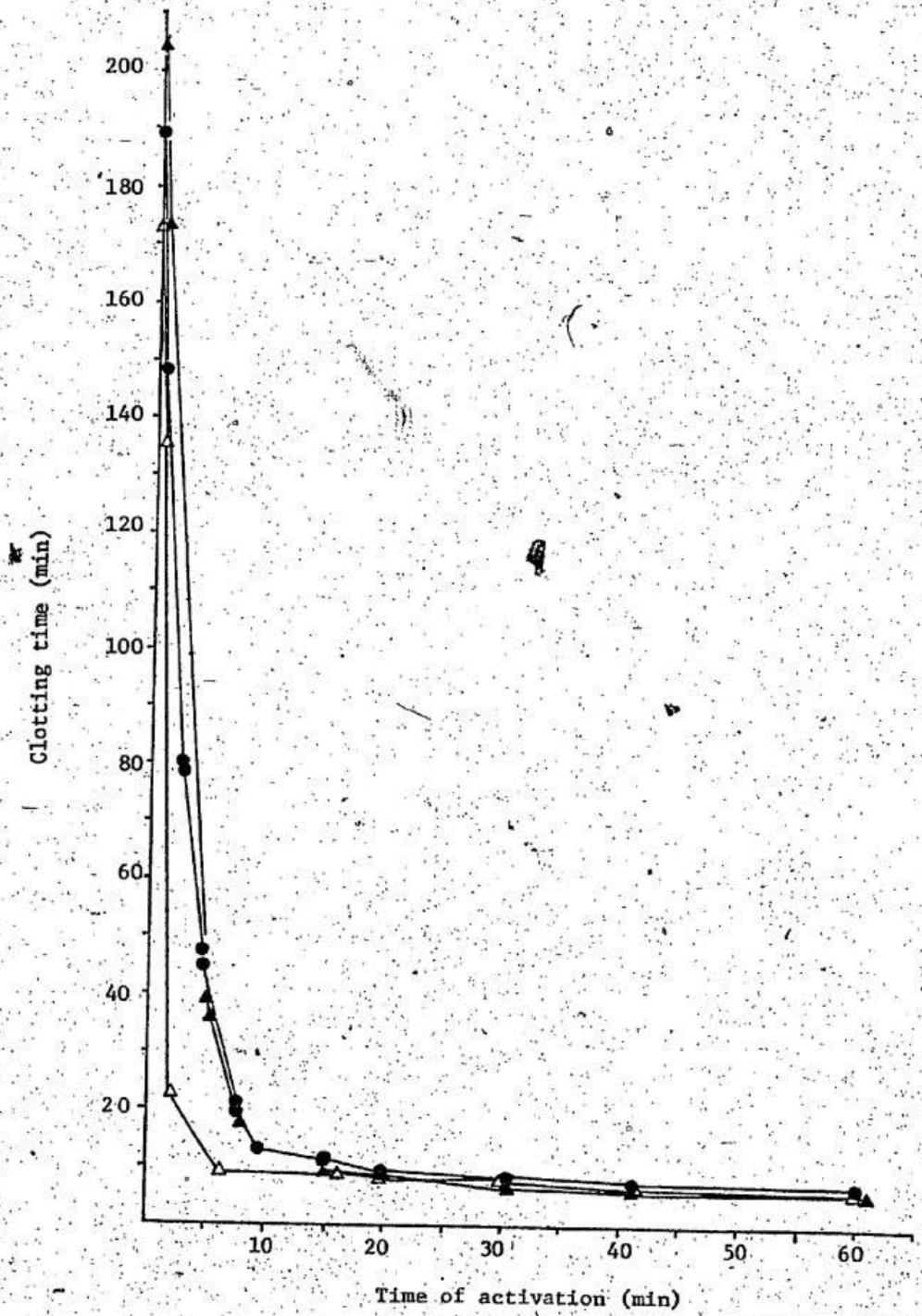
Purification step	Volume (ml)	Activity/ml CU	Activity/ml PU	Total activity CU	Total activity PU	Activity/mg CU	Activity/mg PU	Purification CU	Purification PU	CU/PU
Crude supernatant	46.0	9.0	589.0	414.0	27094.0	1.5	99.8	-		0.015
Chromatography on DEAE Sephadex										
Peak A	6.0	35.5	725.0	213.0	4350.0	10.8	220.0	7.2	2.2	0.049
Peak B	4.4	0.4	14.1	1.8	62.0	0.1	3.9	-	-	0.026
Peak C	6.2	10.9	1457.0	67.6	9033.4	2.6	347.0	1.7	3.5	0.007
Peak C'	4.1	1.6	410.0	6.6	1681.0	0.2	48.8	-	-	0.004
Chromatography on Sephadex G-100										
A	11.4	11.5	153.5	130.5	1750.0	31.8	426.5	21.2	4.3	0.074
C	7.7	3.8	633.2	29.3	4875.6	5.2	867.5	2.5	8.7	0.006
Affinity chromatography										
A	1.9	18.5	250.2	35.2	475.4	39.4	532.4	26.3	5.3	0.075

(c) Summary of purification procedure: Purification steps of the zymogens from the mucosal powder of a two year old harp seal is summarized in Table 2. Zymogen A was purified 21-fold by gel filtration on a Sephadex G-100 column with an increase in its milk clotting activity from 1.5 clotting units/mg of the crude zymogen to 31.5 clotting units/mg. By affinity chromatography of DEAE-peak A a further purification of the milk clotting activity was achieved (39.4 clotting units/mg). Approximately 9 fold purification of zymogen C was achieved by gel filtration; the pepsin activity increased from 99.8 pepsin units/mg of crude extract to 867.5 pepsin units/mg of protein. As a result of purification the ratio of milk clotting activity to proteolytic activity increased in the case of zymogen A compared with this ratio in the crude extract. In the case of zymogen C this ratio decreased by a factor of 4. Under the assay conditions (Sections V(a), V(b)) crystalline calf chymosin had milk clotting and proteolytic activities of 21.4 and 126 units, respectively, per mg of protein based on an average of two determinations.

V. Activation of the Zymogens

The time course of the activation of zymogens A, B and C at 25°C is shown in Figure 5. The pH of an appropriate dilution of the stock solution of each zymogen was adjusted to 2.0 by adding a predetermined volume of 1M HCl. The conversion of the zymogens to the respective enzymes was monitored by milk clotting assay at pH 6.3 (Materials and Methods, Section V(b)). As shown in the figure the activation of zymogen A and B was apparently complete in 15 minutes and that of


1.0 M Acetate pH 3.5
1.0 M GuHCl pH 3.5
0.2
0.1
A280 nm
0.6
0.4
0.2
Proteolytic Activity
10
20
30
40
Fraction Number


Clotting time (min)
200
180
160
140
120
100
80
60
40
20
10
20
30
40
50
60
Time of activation (min)

zymogen C in 6 minutes. These observations served as a guideline to activate the zymogens prior to routine assays. Routinely the activation process was carried out for 30 minutes.

VI. Properties of the enzymes

(a) CU/PU ratios: The ratios of milk clotting activity to proteolytic activity of the crude isoenzyme forms A, B, C and C' are shown in Tables 3 and 4. The ratios for enzyme A were 6 to 9 fold higher than that of the other major isoenzyme C. However, as shown in Table 2, the ratio for the purified zymogen A was about 12 times as high as that of purified zymogen C. The CU:PU ratios for isoenzyme B were similar to those of A and the ratios for isoenzyme C' are similar to those for isoenzyme C irrespective of the age of the animals.

The CU:PU ratios for the total extract from the two-week-old pups were slightly higher than those for the crude enzymes from the adult seals as shown in Table 1. This is consistent with the higher proportion of isoenzyme A in the two-week-old pup stomachs. The CU:PU ratio for protease A purified by gel filtration and affinity chromatography was higher (Table 2) than that of the crude isoenzyme A (Tables 3 and 4). This suggests that the purification process removed some material(s) with low CU:PU ratio. It is also possible that some other nonenzymatic factors which selectively affect the milk clotting activity of enzyme A were removed by the purification.

(b) Milk clotting as a function of pH: Milk clotting times for active isoenzymes A and C are shown in Figure 6a and that of isoenzyme B is shown in Figure 6b. Clotting times for porcine pepsin and calf

Table 3. Relative rates of milk-clotting and hemoglobin hydrolysis by various DEAE peaks from homogenate of 2-week-old harp seal gastric mucosa

	Peak	Pepsin Unit/mg	Milk-Clotting Unit/mg[2]	$\frac{CU}{PU}$
Experiment 1	A	278.0	14.2	0.051
	B	13.08	0.69	0.053
	C	223.8	1.83	0.008
	C'	1.9	0.02	0.007
Experiment 2[3]	A	104.6	7.48	0.072
	B	6.6	0.36	0.056
	C	118.9	1.01	0.009
	C'	1.6	0.01	0.006

1. Peptic activity was determined at 3 to 4 different concentrations.
2. Average of at least two determinations.
3. The freeze-dried powder was stored in a -20°C freezer for about a year before the extraction. Some activity may have been lost during storage.

Table 4. Relative rates of milk-clotting and hemoglobin hydrolysis by DEAE peaks from homogenates of 2-year-old harp seal gastric mucosa

	Peak	Pepsin Unit/mg[1]	Milk-Clotting Unit/mg[2]	$\frac{CU}{PU}$
Experiment 1	A	219.6	10.5	0.048
	B	3.9	0.12	0.031
	C	346.9	3.09	0.009
	C'	48.8	1.6	0.004
Experiment 2	A	228.0	12.6	0.055
	B	9.70	.46	0.047
	C	230.2	1.71	0.007
	C'	46.5	0.28	0.006

1. Pepsin activity was determined at 3 to 4 different concentrations of each sample in duplicate.
2. Average of at least two determinations.

Figure 6. Milk-clotting activity as a function of pH.

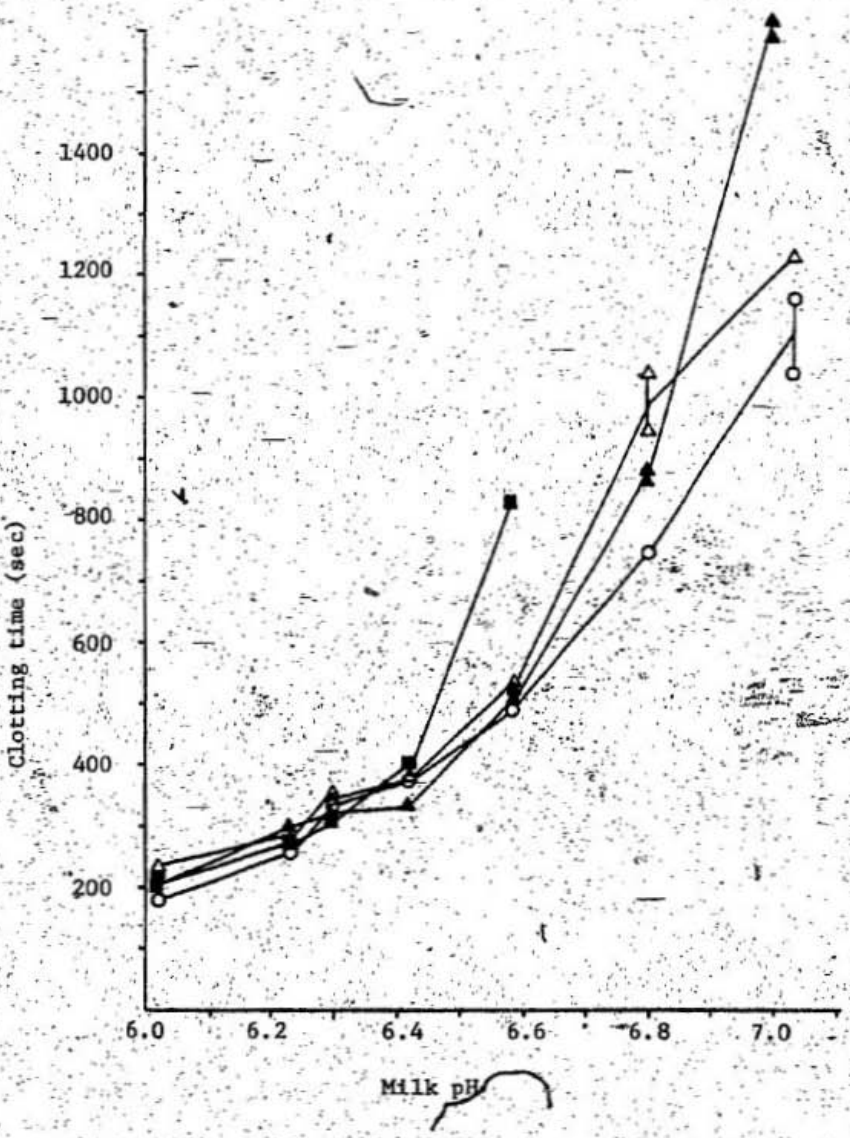
A 12% suspension of skim milk in 0.01M $CaCl_2$ was adjusted to pH 6.0 to 7.0.2 with 1M HCl or 1M NaOH. The concentration of each enzyme was adjusted so that a 50 μl volume will clot milk at pH 6.3 in 5 minutes $\pm$ 10 seconds.

(a) Milk-clotting activity of proteases A, and C compared with those of calf chymosin and porcine pepsin.

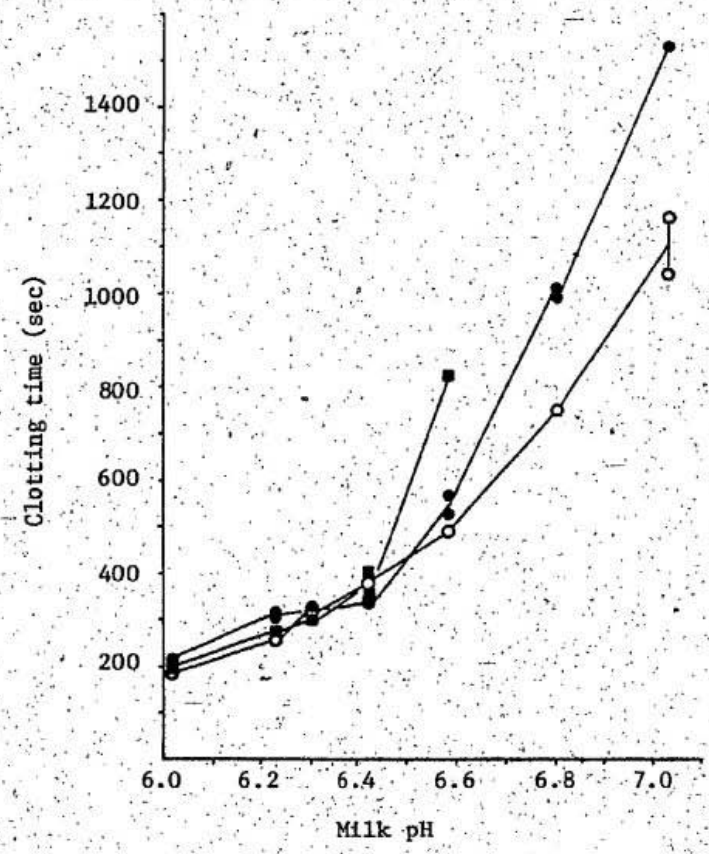
(b) Protease B compared with porcine pepsin and calf chymosin.

○, calf chymosin; ■, porcine pepsin; ▲, protease A; △, protease C; ●, protease B.

(a)


1400
1200
1000
800
600
400
200
Clotting time (sec)
6.0
6.2
6.4
6.6
6.8
7.0
Milk pH

(b)


Clotting time (sec)
1400
1200
1000
800
600
400
200
6.0
6.2
6.4
6.6
6.8
7.0
Milk pH

chymosin are also included for comparison. The three seal gastric proteases exhibited a pH dependency for clotting times similar to that for calf chymosin and different from that of porcine pepsin.

Anifantakis and Green (1980) observed that the milk clotting activities of lamb and kid rennets increased less with decrease in pH than that of calf rennet.

(c) Action of proteases on ribonuclease: The inactivation of bovine pancreatic ribonuclease by the proteolytic enzymes was performed as described in Materials and Methods (Section V(d)). Incubation with seal proteases A, B and the crude enzyme was carried out at pH 2.6 based on the pH optimum of crude seal gastric proteases (Appendix D). Incubation with porcine pepsin and seal protease C was carried out at pH 2.0 and that with calf chymosin was performed at pH 3.5. As shown in Table 5 there was no detectable inactivation of ribonuclease by the proteases A, B or calf chymosin after 2 hours of incubation whereas protease C caused an average of 18% inactivation. Porcine pepsin at the potencies of 1.6 and 5.8 pepsin units effected 55% and 84% inactivation, respectively.

Inactivation of ribonuclease by crude SGP was also examined. Figure 7 shows the percentage losses of ribonuclease activity after 2 h incubation with various concentrations of crude SGP and of porcine pepsin at 30°C. The figure shows that with 76 pepsin units of enzyme, seal gastric protease inactivated only 22% of the ribonuclease activity whereas porcine pepsin with the same potency inactivated 79% of the ribonuclease.

Table 5. Inactivation of ribonuclease

Protease	Potency (Pepsin unit)	pH of incubation	% inactivation[1]
DEAE Peak A	11.8	2.6[2]	0
Peak B	1.6	2.6[2]	0
Peak C	6.1	2.0	18
Porcine pepsin	1.6	2.0	55
	5.8	2.0	84
Calf chymosin	6.0	3.5	0

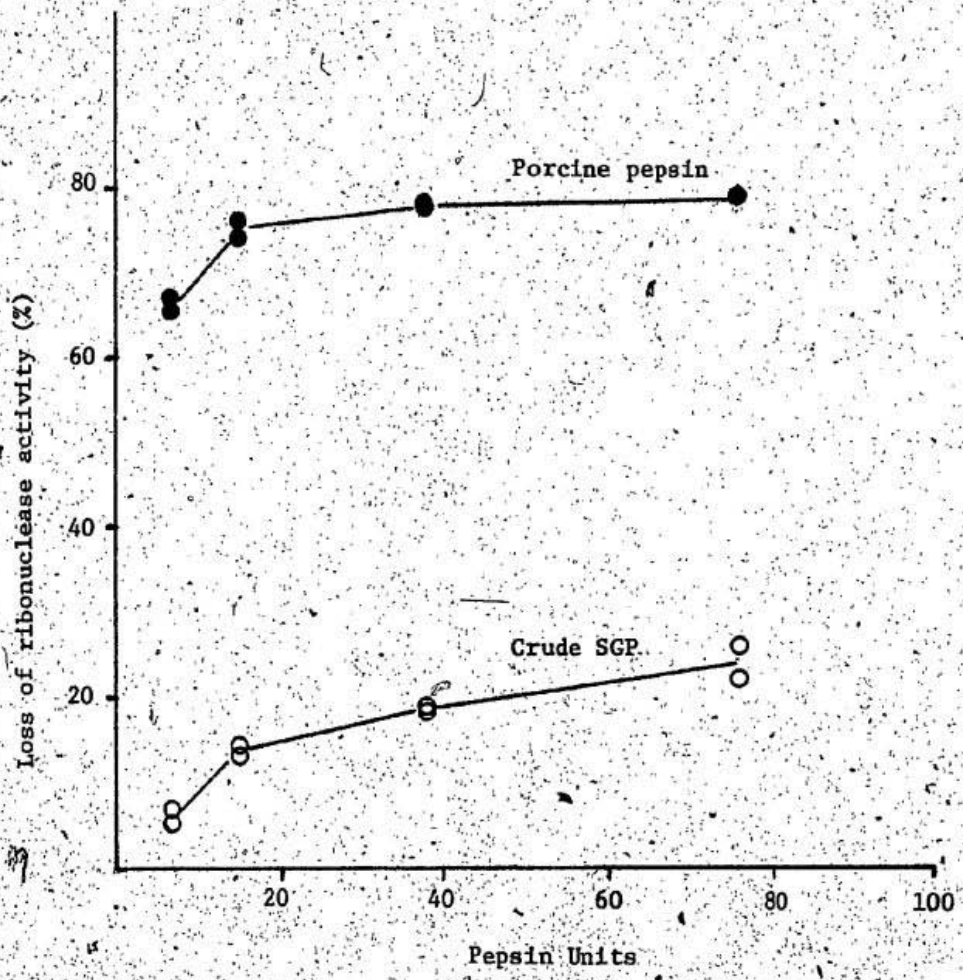
[1] Average of duplicate measurements.

[2] Based on the pH optimum for the pepsin activity of crude SGP.

Figure 7. Inactivation of ribonuclease by crude SGP.

An active preparation of a crude extract from adult-seal mucosa was diluted so that 0.1 ml would contain the pepsin units indicated in the figure. After 2 hours of incubation with ribonuclease the residual ribonuclease activity was determined as described in Materials and Methods. Incubation with porcine pepsin was at pH 2.0 and that with crude SGP at pH 2.6.

○, crude SGP; ●, porcine pepsin.


Porcine pepsin
Crude SGP
Loss of ribonuclease activity (%)
80
60
40
20
20
40
60
80
100
Pepsin Units

(d) Hydrolysis of APDT: Hydrolysis of the pepsin substrate N-acetyl-L-phenyl-alanyl-L-diiodotyrosine (APDT) by protease A was very low. As shown in Table 6 the activity of protease A expressed as the ratio of APDT unit/pepsin unit of the enzyme was similar to that of calf chymosin, but much lower than those of protease C and porcine pepsin. The activity of protease B on this substrate was negligible. Very high concentrations of proteases A and calf chymosin were used in order to obtain detectable increases in absorbance at 570nm. As a result of this, there was high background absorbance and during the initial 2h incubation the increase in A570nm was very low and reproducibility was poor. However, there was appreciable increase in absorbance after 4 h and 13 h of incubation. The relatively low activity of protease A and calf chymosin on APDT shown in this study is consistent with reports that chymosin is much less sensitive to certain synthetic substrates than to κ-casein and milk (Martin et al., 1981). These authors also showed that bovine pepsin A was 55 fold as active on the hexapeptide Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe than calf chymosin when equal milk clotting units of the two enzymes were used.

(e) Clotting activity as a function of temperature: Clotting activity of calf chymosin and protease A as a function of temperature is shown in Figure 8a. Purified peak A protease had milk-clotting times close to those of calf chymosin in the temperature range 15° to 45°C. At 50°C the clotting time for protease A was higher than that for calf chymosin.

Arrhenius plots for the milk-clotting activities at various

Table 6. Hydrolysis of N-acetyl-L-phenyl alanyl-L-diiodotyrosine (APDT)

Protease	Potency (PU)	Incubation time (h)	Absorbance (570nm) 2	4	13	$AU^1/PUx10^4$
A	11.8		0.06±0.06	0.063±0.007	0.222±0.009	2.82
B	1.6		0.055±0.055	-0.02±0.02	0.08±0.08	—[3]
C	6.1		0.015±0.015	0.172±0.023	0.275±0.028	14.9
Porcine pepsin	1.6		0.065±0.001	0.106±0.005	0.171±0.001	42.9
Calf chymosin	8.8		0.017±0.04	0.073±0.011	0.297±0.015	4.4

1. One APDT unit (AU) is the amount of enzyme that causes an absorbance increase at 570nm of 0.079/min. Because of high background absorbance and low activity readings after 2h were not reliable for A, C, and calf chymosin. Therefore values of $A_{570}{}^{nm}$ after 4h were used for calculation of AU in these cases.

2. Average of duplicate readings ± range after substracting the A_{570} of control samples.

3. Could not ascertain because of low APDT activity.

temperatures for calf chymosin and protease A are shown in Figure 8b. Activation energies of 13.1 and 15.6 Kcal/mole were calculated for chymosin and protease A respectively. The calculation was based on the slopes of an Arrhenius plot computed from linear regression of the points in the temperature range 15° to 35°C. Above that range data points were scattered in the case of protease A. In the case of calf chymosin there were breaks in the Arrhenius plot, one at 20°C and the other at 35°C. This may relate to the heterogeneity of the chymosin as will be shown later (see Figure 17). Milk clotting activities of chicken pepsin and calf chymosin as a function of temperature reported by Gordin and Rosenthal (1978) were also plotted here for comparison. The energies of activation for chicken pepsin and calf rennet calculated from Gordin and Rosenthal's data (1978) are 15.0 and 11.6 Kcal/mole respectively.

It should be noted that the activation energy for the milk clotting activity of an enzyme is a complex property because milk clotting is a two-stage process and the temperature dependence of the nonenzymatic step is different from that of the enzymatic step (Dalgleish, personal communication).

(f). Influence of Ca^{++} on clotting activity: Milk clotting activity of protease A was found equally sensitive to added calcium chloride concentration as calf chymosin (Figure 9). Highest activity was found at 0.02M Ca^{++} for both the enzymes; at higher concentrations activity was inhibited. At 0.1M calcium chloride concentration the clotting times were similar to those without added calcium chloride.

The stimulation of milk clotting activity of coagulants by added

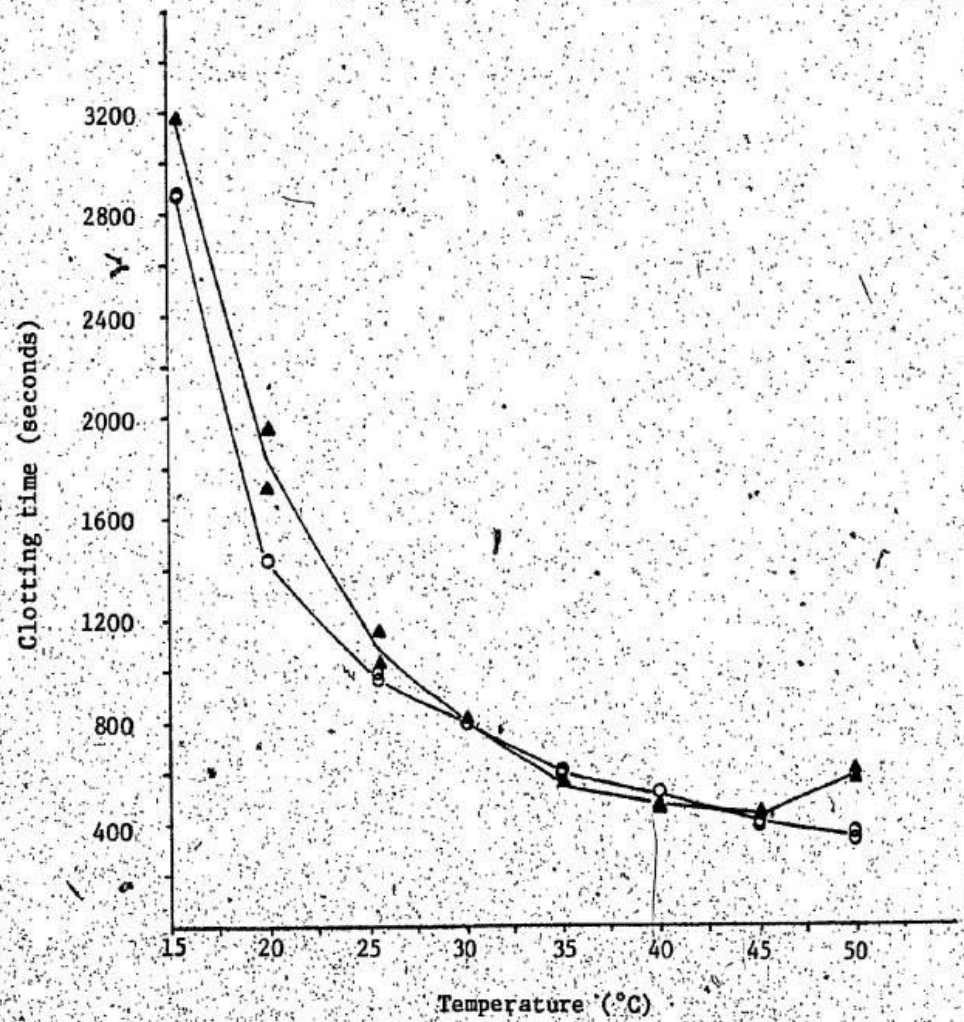
Figure 8. Effect of temperature on clotting activity.

Concentrations of enzymes were so adjusted that both calf chymosin and Enzyme A had equal milk clotting activity at 30°C in Berridge substrate pH 6.3.

(a) clotting times as a function of temperature for calf chymosin (○), protease A (△).

(b) Arrhenius plots calculated from this experiment, ○---○ calf chymosin; ▲, protease A. Arrhenius plots calculated from data reported by Gordin and Rosenthal (1978) are shown as ○—○ calf chymosin; ●—● chicken pepsin.

(a)


Clotting time (seconds)
3200
2800
2400
2000
1600
1200
800
400
15
20
25
30
35
40
45
50
Temperature (°C)

(b)

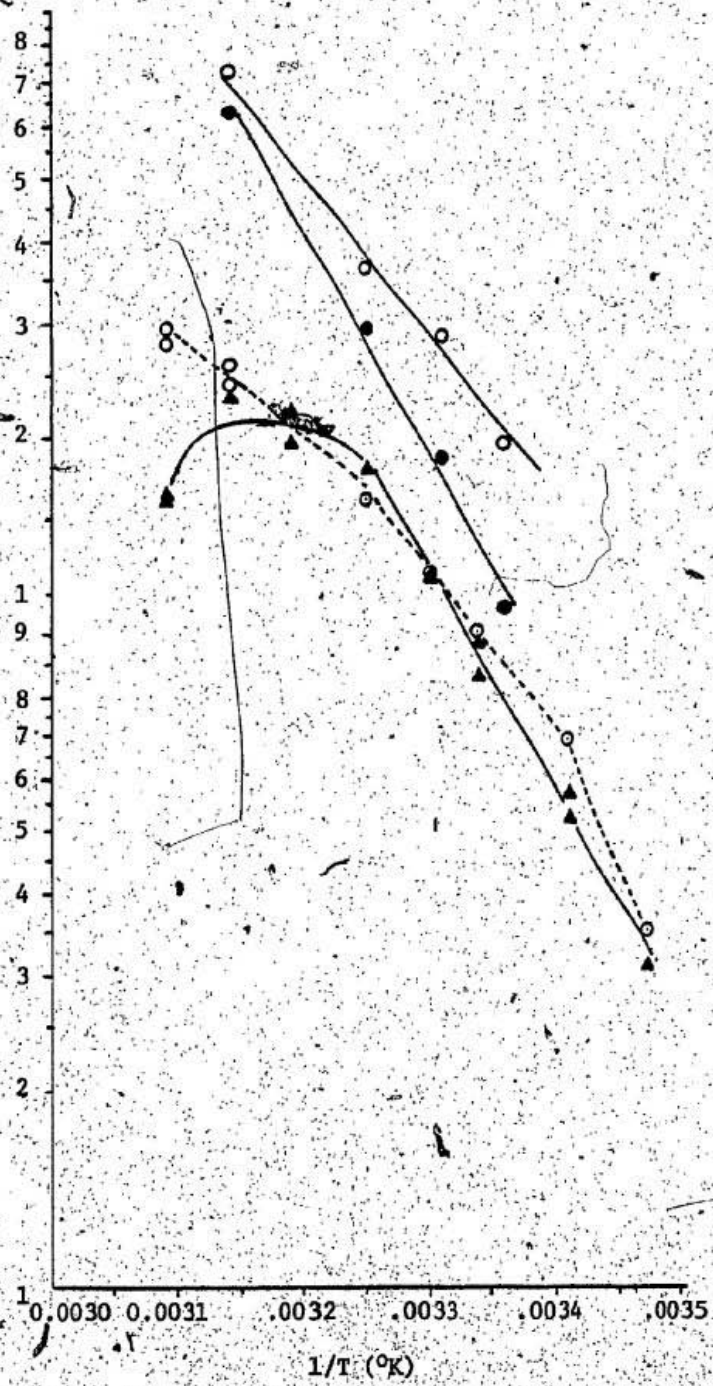
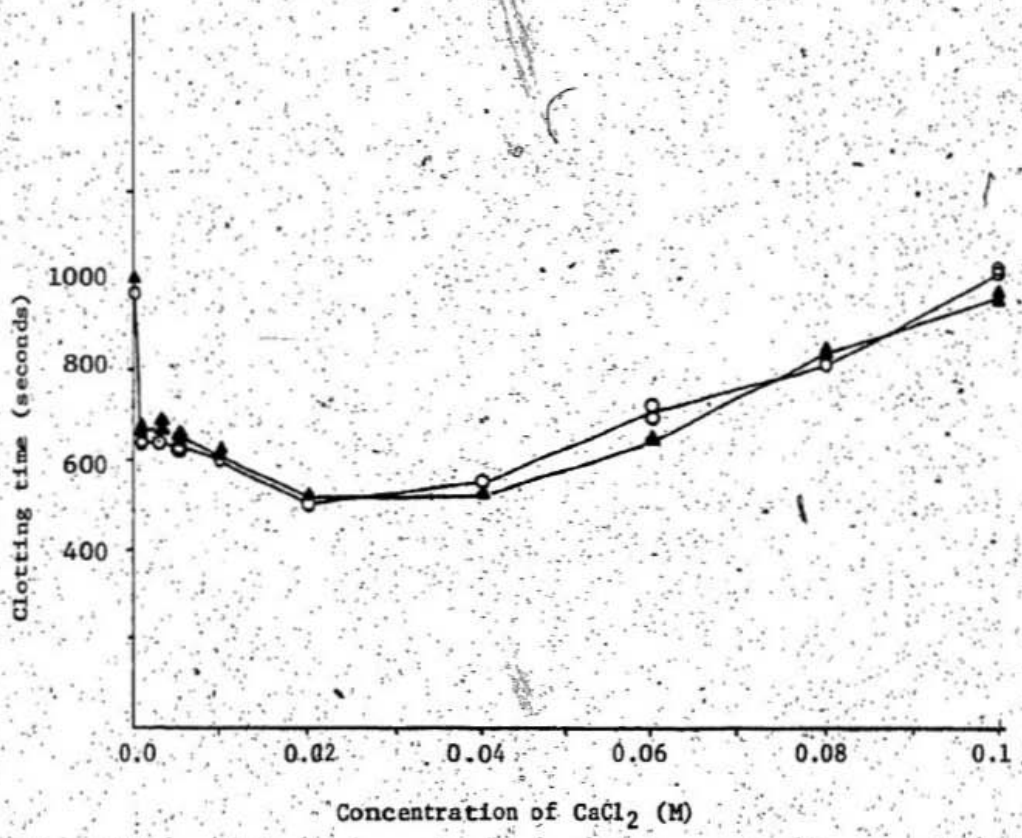

Inverse clotting time (second)$^{-1}$ x 10^5
8
7
6
5
4
3
2
1
9
8
7
6
5
4
3
2
1
0.0030
0.0031
.0032
.0033
.0034
.0035
1/T (°K)

Figure 9. Influence of calcium chloride concentration on milk-clotting activity.

Suspensions of 12% skim milk were made at various levels of calcium chloride concentrations at pH 6.3. Concentration of each of the enzymes was adjusted so that they had equal clotting time at 0.01M $CaCl_2$ concentration.

O, calf chymosin; ▲, protease A.

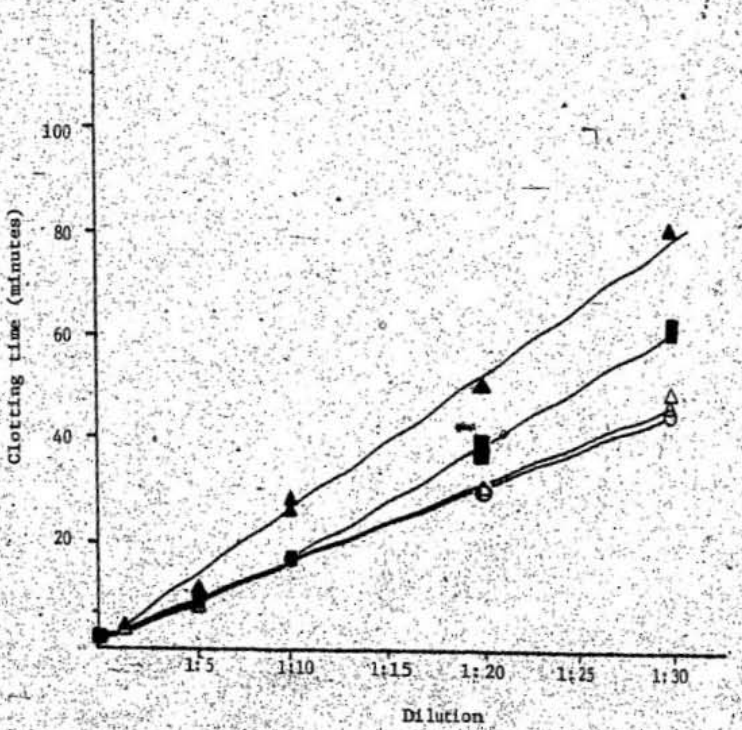

Clotting time (seconds)
1000
800
600
400
0.0
0.02
0.04
0.06
0.08
0.1
Concentration of $CaCl_2$ (M)

calcium observed in this study is in agreement with reports of Gordin and Rosenthal (1978) and of Tsugo and Yamauchi (1959). However these authors observed only small decreases in the clotting activities of calf rennet and other coagulants at high $CaCl_2$ concentration compared with the decrease in clotting activities of protease A and calf chymosin in this study. These discrepancies are probably due to differences in the conditions of milk-clotting assay. The drop in milk pH that occurs on the addition of $CaCl_2$ was not adjusted in the studies reported by these authors. The pH of reconstituted skim milk in 0.01M $CaCl_2$ (Berridge substrate) used routinely in this study was 6.3 and its pH dropped to 5.5 on addition of 0.1M $CaCl_2$. The delay in the clotting times due to the addition of $CaCl_2$ was probably offset by the lower pH of the milk used by Gordin and Rosenthal (1978). However, it is possible that part of the inhibition of milk-clotting activities observed in this study was due to irreversible change in the casein micelles as a result of pH adjustment as suggested by Gordin and Rosenthal. Amer et al. (1980) reported slight stimulation of milk-clotting activity of bovine chymosin and pepsin and inhibition of their proteolytic activity by $CaCl_2$ and NaCl.

(g) Clotting time as a function of enzyme concentration: Milk-clotting times increased linearly with dilution of both protease A and calf chymosin as shown in Figure 10, but the increase was higher in the case of protease A than the increase in clotting times for calf chymosin at the same dilution. Porcine pepsin also had a steeper slope than that of calf chymosin whereas protease C had similar activity with dilution as calf chymosin.

Figure 10. Effect of enzyme dilution on the clotting activity.

Enzyme concentration was adjusted to clot 1 ml of Berridge substrate in 100 ± 10 seconds. This stock enzyme was then diluted with 10mM sodium acetate at pH 5.3 and immediately 50 µl mixed with 1 ml of Berridge substrate equilibrated at 30°C, Calf chymosin,○; Protease A,▲; Protease C,△; and Porcine pepsin, ■.

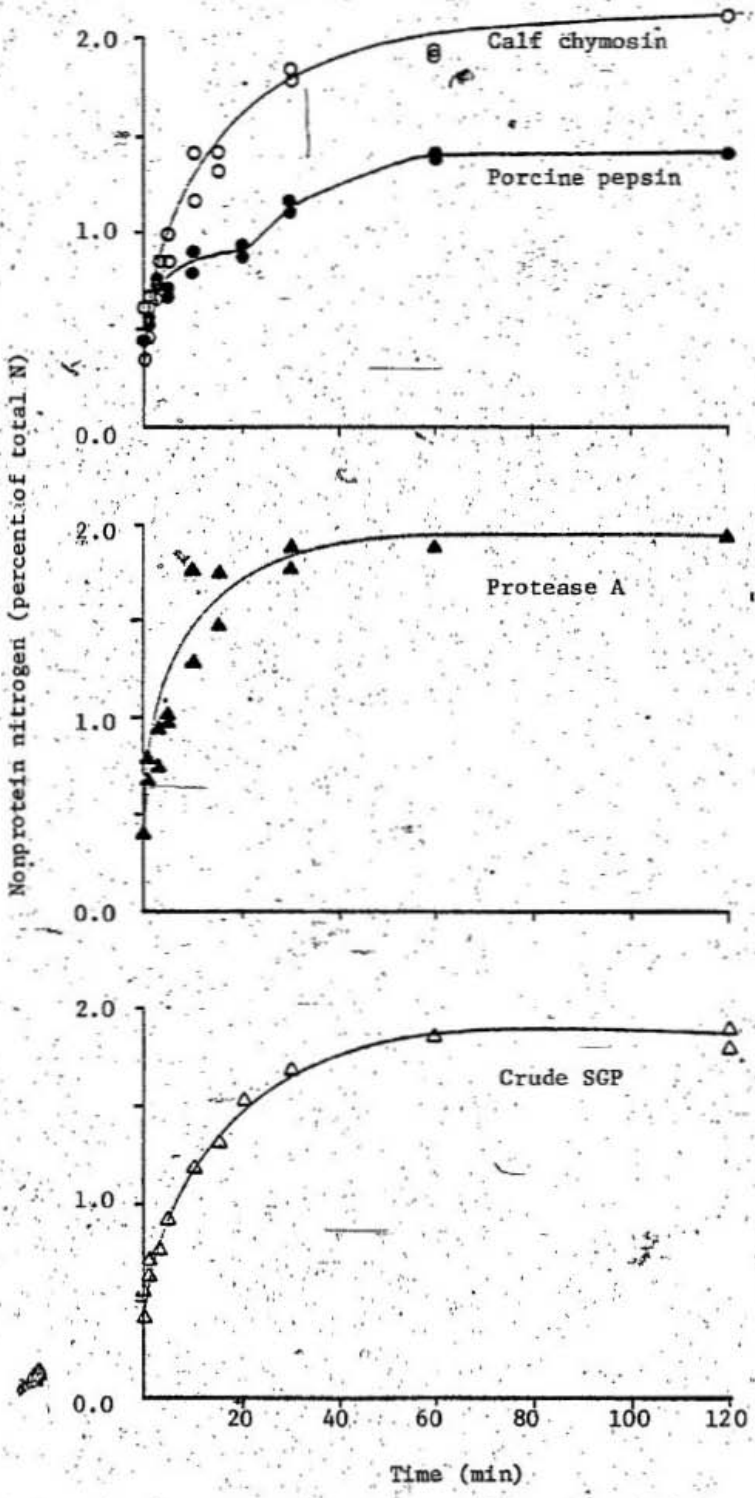

Clotting time (minutes)
100
80
60
40
20
1:5
1:10
1:15
1:20
1:25
1:30
Dilution

(h) Hydrolysis of casein: The extent of hydrolysis of casein by the coagulants was examined at the enzyme concentrations ordinarily used for cheesemaking. Forty ml of a 2% casein solution pH 6.1 was incubated with 2.0 ml of the enzyme at 30°C. The concentrations of the enzymes were standardized so that 2.0 ml volume of the enzymes would clot 40 ml of milk in approximately 20 minutes. At intervals, samples were withdrawn and nonprotein nitrogen determined as described in the legend to Figure 11. As shown in the figure the release of nonprotein nitrogen due to hydrolysis by protease A and crude SGP with time was similar to that of calf chymosin. In both the cases no further hydrolysis was detectable beyond the 1.7% NPN expressed as percentage of total nitrogen, taking into account the 0.3% NPN already present in the samples at 0 time. With porcine pepsin however, the release of nonprotein nitrogen leveled off at 1%. The release of NPN from casein by chymosin (Alais et al., 1953; Wake, 1959) has been correlated to the action of chymosin specifically on the κ-casein fraction which comprises 10–20% of whole casein. A mixture of macropeptides is released as a result (Wake, 1959; Alais and Jolles, 1961) part of which is soluble in 12% TCA. Clotting of milk by bovine, porcine, and chicken pepsins (Green, 1972) and microbial protease (Ohmiya et al., 1979) also involves the same mechanism. However, with non-rennet milk coagulants there is a rapid release of NPN followed by small but gradual increase in NPN indicating nonspecific proteolysis as with chicken pepsin (Gordin and Rosenthal, 1978) and Mucor pulilus protease (Pahkala and Antila, 1980).

(i) Influence of pH on hemoglobin hydrolysis: Effect of pH on the

Figure [illegible] Hydrolysis of casein by proteolytic enzymes.

Forty ml of a 2% solution of casein, pH adjusted to 6.1, was equilibrated at 30°C. To this was added 0.2 ml enzyme calculated to clot 40 ml of milk in 20 minutes. At intervals shown in the figure 2 ml samples were withdrawn in duplicate, precipitated by adding 2 ml of 24% TCA. Centrifuged at 3000xg for 30 minutes. One ml aliquot of the supernatant was subjected to N determination following the method of Lang (1958). The nonprotein nitrogen was expressed as percentages of total N. The initial NPN present in the samples was determined by taking an aliquot before adding the enzymes.


Calf chymosin
Porcine pepsin
Protease A
Crude SGP
Nonprotein nitrogen (percent of total N)
2.0
1.0
0.0
20
40
60
80
100
120
Time (min)

rate of hemoglobin hydrolysis at 30°C by proteases A and C are shown in Figure 12. Peak C protease had an optimum pH at 2.4 and protease A showed activity over a wide pH range between 2.2 and 3.5. In this respect protease A resembles calf chymosin (Berridge, 1945) and gastricsin (Ward et al., 1978).

(j) Influence of pH on enzyme stability: The stability of the active enzymes A and C was compared in buffers of various pH values having an ionic strength 0.05 after 24 hours of incubation at 25°C. At pH values below 7.0 protease A lost more pepsin activity than did the other proteases (Fig. 13). At pH 7.0 it was much more stable than porcine pepsin and less stable than calf chymosin and protease C. These observations support those summarised in Figures 6a, 6b. In the acidic pH range all the proteases lost more activity at their pH optima for hemoglobin hydrolysis than at any other pH values presumably because of autodigestion (Foltmann, 1959). All the proteases showed minimum loss of activity between pH 5.0 and 6.0.

(k) Denaturation of proteases by urea: The denaturation of protease A and C in 6M urea at pH 5.40 and 37°C was compared with that of calf chymosin, and porcine pepsin. As shown in Figure 14, after 90-minute incubation both protease A and calf chymosin were rapidly denatured and lost about 90% of their initial pepsin activity. The initial rate of denaturation of protease A was considerably faster than that of the other proteases. Seal protease C and porcine pepsin lost 62% and 56% of their original activity respectively, in 90 minutes. These results agree with those of Cheeseman (1965) who observed only 13% inactivation

Figure 12. Effect of substrate p^H on proteolytic activity.

Hemoglobin solutions of the p^H values indicated in the figure were prepared as described in Materials and Methods and incubated at 30°C for 60 minutes with 20 ul of each enzyme containing 0.4 pepsin unit. The ionic strength of the buffers was 0.05.

▲, Protease A; Δ, protease C.

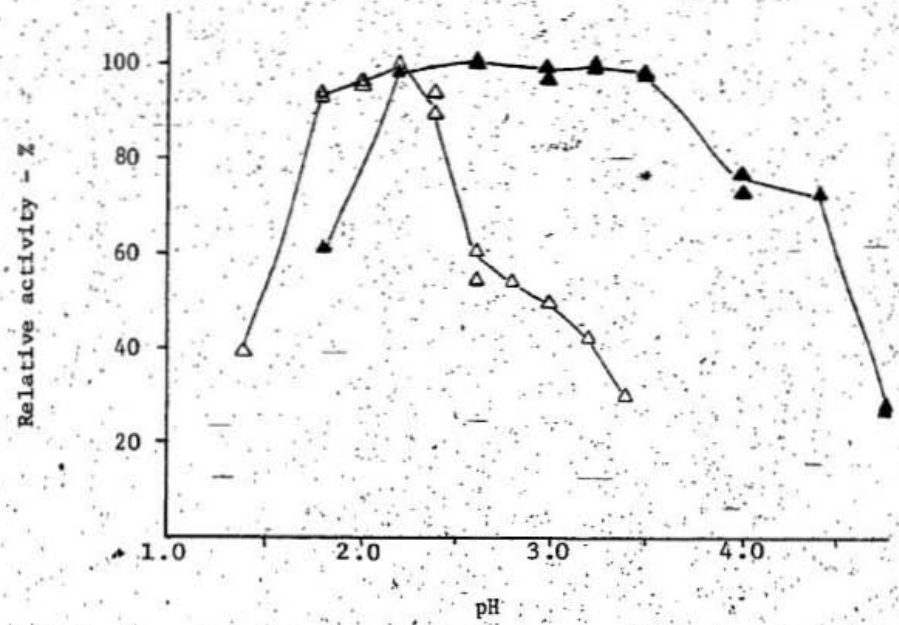

Relative activity - %
100
80
60
40
20
1.0
2.0
3.0
4.0
pH

Figure 13. Stability of the proteases with respect to pH.

To 0.5 ml of enzyme solutions 0.5 ml of buffers were added to give the final pH values indicated in the Figure. The mixtures were incubated at 25°C. After 24h residual proteolytic activities were determined as described in Materials and Methods. ○, Citrate; □, acetate; and Δ, phosphate buffers.

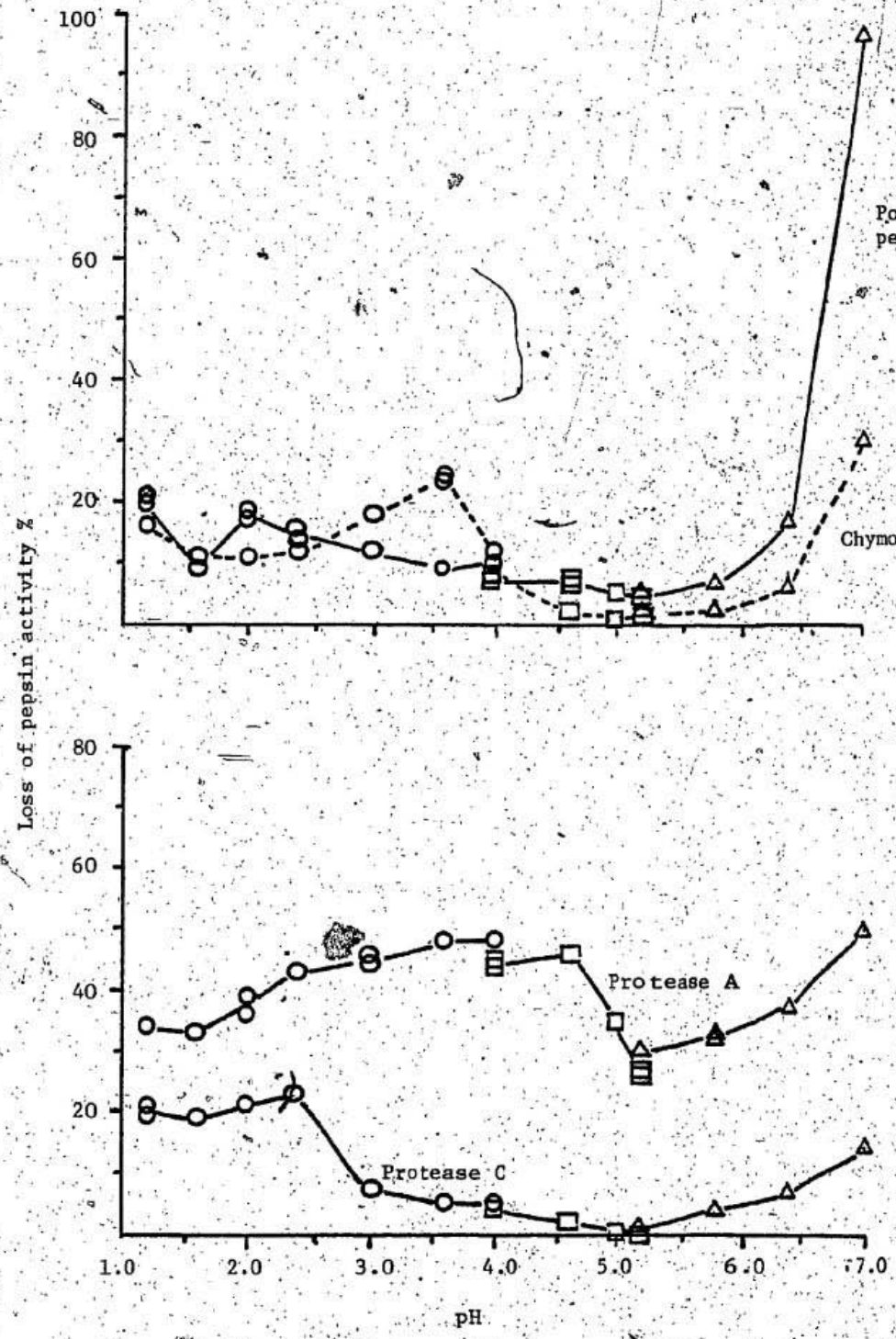
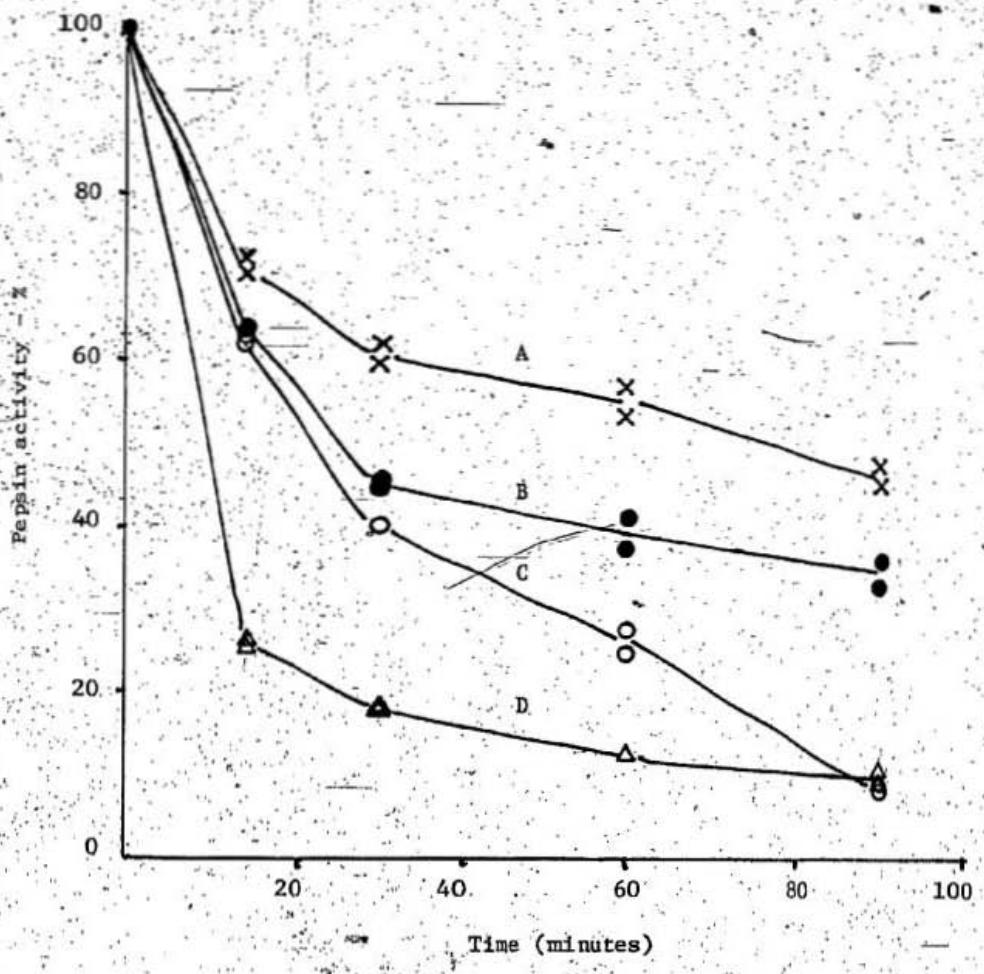

100
80
60
40
20
Porcine pepsin
Chymosin
Loss of pepsin activity %
80
60
40
20
Protease A
Protease C
1.0
2.0
3.0
4.0
5.0
6.0
7.0
pH

Figure 14. Inactivation of proteases in 6M urea.

To 0.4ml of 9M urea in 0.15M sodium acetate equilibrated at 37°C, 0.2 ml solution of the enzymes was added, so that the final concentration of urea was 6M at pH 5.4. The mixture was then incubated at 37°C and at indicated intervals 30 µl aliquots were withdrawn and the residual hemoglobin hydrolytic activities determined as described in Materials and Methods.

A, protease C; B, porcine pepsin; C, calf chymosin; and D, protease A.

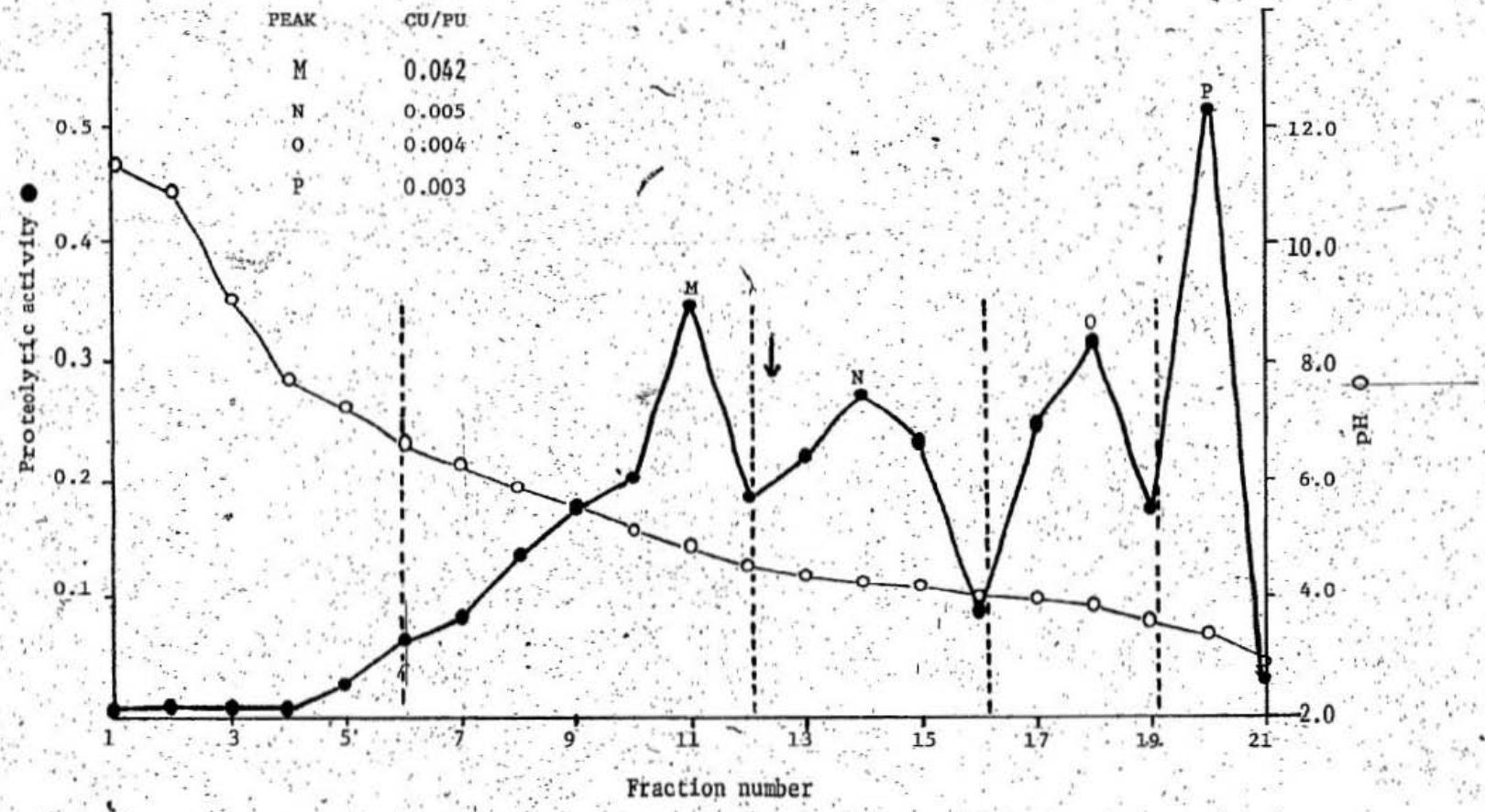

100
80
60
40
20
0
Pepsin activity - %
A
B
C
D
20
40
60
80
100
Time (minutes)

of pepsin whereas purified calf chymosin B lost 90% of its initial activity in 90 minutes. There were not enough details about the experimental procedures in Cheeseman's report apart from pH, molarity of urea and incubation temperature. Therefore the difference in the degree of inactivation of pepsin in this study from that found by Cheeseman may be attributed to any possible differences in the experimental conditions, e.g. type and concentration of buffer and the purity of pepsin used.

(1) Isoelectric points: A crude preparation of proteases from an adult harp seal stomach resolved into 4 isoenzymes by preparative isoelectric focusing on Sephadex IEF (Figure 15). The isoelectric points of the isoenzymes, M, N, O and P were 4.90, 4.31, 3.82 and 3.40 respectively. The result of isoelectric focusing agrees with the separation of four zymogens by ion-exchange chromatography on DEAE-Sephadex column. However, the relative peak sizes of the proteases M, N, O, and P are different from those of the zymogens A, B, C and C' separated by DEAE-Sephadex chromatography. It is possible that the recoveries of zymogens B and C' from the DEAE-Sephadex column were less than those of the zymogens A and C. It was not determined which isoenzyme separated by isoelectric focusing corresponds to which zymogen eluted from the DEAE-Sephadex column. However, isoenzyme M is probably the same as protease A since they had similar CU:PU ratio. The isoelectric point of calf chymosin was reported to be 4.70 $\pm$ 0.05 (Righetti et al., 1977) similar to that of the seal isoenzyme M. The isoelectric point of purified zymogen C was 4.02 by analytical polyacrylamide gel isoelectric focusing. No protein band

Figure 15. Preparative isoelectric focusing of crude SGP on sephadex IEF.

Five hundred µl of crude proteases from an adult harp seal stomach were subjected to isoelectric focusing as described in Materials and Methods (section VIII). The sample was applied in the position indicated by the arrow. After electrofocusing the protein was eluted from the gel fractions and proteolytic activity determined as described in Materials and Methods. Fractions with proteolytic activity were divided as indicated by dotted lines and pooled to determine CU:PU.


PEAK
CU/PU
M
0.042
N
0.005
O
0.004
P
0.003
Proteolytic activity ●
pH
Fraction number
0.5
0.4
0.3
0.2
0.1
12.0
10.0
8.0
6.0
4.0
2.0
1
3
5
7
9
11
13
15
17
19
21
M
N
O
P

was visible when isoelectric focusing was performed on purified zymogen A under the same conditions. The protein (9 µg in 20 µl) in the sample probably separated into fractions below detectable level.

(m) Purity of the proteases: Zymogen A purified by gel filtration on a Sephadex G-100 column emerged as a single symmetrical peak by high-performance liquid chromatography on a column of Beckman spherogel-TSK3000SW (Figure 16). However, on polyacrylamide gel electrophoresis at pH 8.3 (Davis, 1964) zymogen A appeared as a broad smear covering an area between Rf 0.17 to 0.45 (not shown) and a very faint hazy band between Rf 0.46 and 0.63. Zymogen C, however, was electrophoretically homogeneous with an Rf 0.713. After activation of zymogen C at pH 2.0 its mobility was lower than that of the zymogen (Rf 0.610), as shown in Figure 17. DEAE-peak A zymogen after purification by affinity chromatography on Z-D-Phe-T-Sepharose column was also electrophoretically heterogeneous (Figure 18). It moved as two closely moving broad bands with Rf's 0.39 (B) and 0.50 (C). There were also two smaller bands with Rf's 0.07 (A) and 0.63 (D). Since the affinity chromatography was done at pH 3.5 it was likely that the electrophoretic heterogeneity was due to slow conversion of zymogen A to protease A and its possible autodigestion. To examine this possibility zymogen A was incubated at pH 2.0 for 30 minutes for complete activation. On electrophoresis of this protease A it was found that the band A disappeared and two smaller bands a_1 and a_2 preceeded the band B. The absorbance of band B was decreased to 1.1 from 1.3 optical density units on activation. The area under peak B for the zymogen decreased from 53% to 35% after activation. Peak C increased in area

Figure 16. Chromatography of Zymogen A on high-pressure liquid chromatography.

A 20 µl volume of zymogen A (15 µg protein) purified on Sephadex G-100 column was subjected to high-performance liquid chromatography on a Beckman model 334 HPLC system with a Hitachi variable wavelength detector and a 7.5 x 600 mm spherogel-TSK 3000 SW column (Beckman Instruments). The column was previously calibrated with bovine serum albumin (65,000 daltons), chicken egg albumin (43,000 daltons), myoglobin (17,000 daltons), lysozyme (14,500), and cytochrome C (12,700 daltons). Flow rate, 1 ml/min.

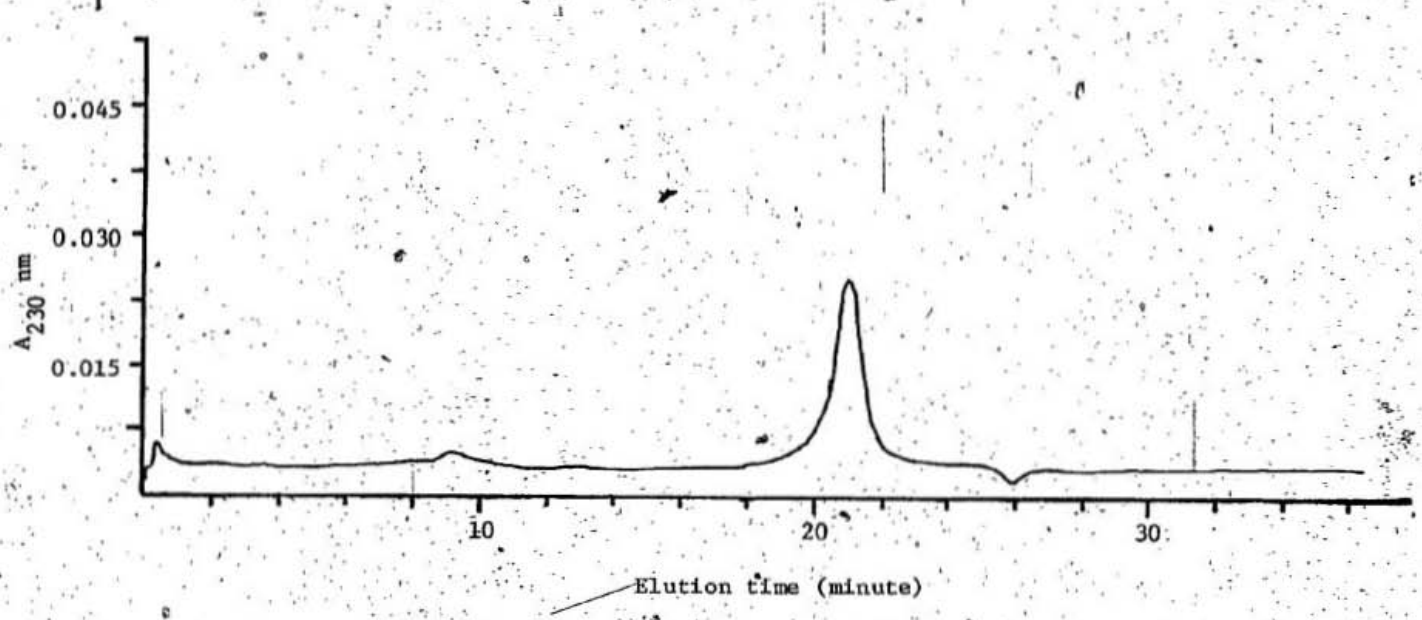

0.045
0.030
0.015
$A_{230\ nm}$
10
20
30
Elution time (minute)

Figure 17. Electrophoresis of zymogens and enzymes.

Polyacrylamide gel electrophoresis was carried out at pH 8.3 following the method of Davis (1964) at 125V constant-voltage setting of a Pharmacia ECPS 2000 power supply for 6 hours with cold-water circulation maintained at 4°C. The gels were cut off at dye front after electrophoresis, stained for 30 minutes in 0.1% Coomassie blue and destained by diffusion in solution containing 5% methanol, and 10% acetic acid. 1, zymogen C, 2, protease C, 3, porcine pepsin, 4, calf chymosin.

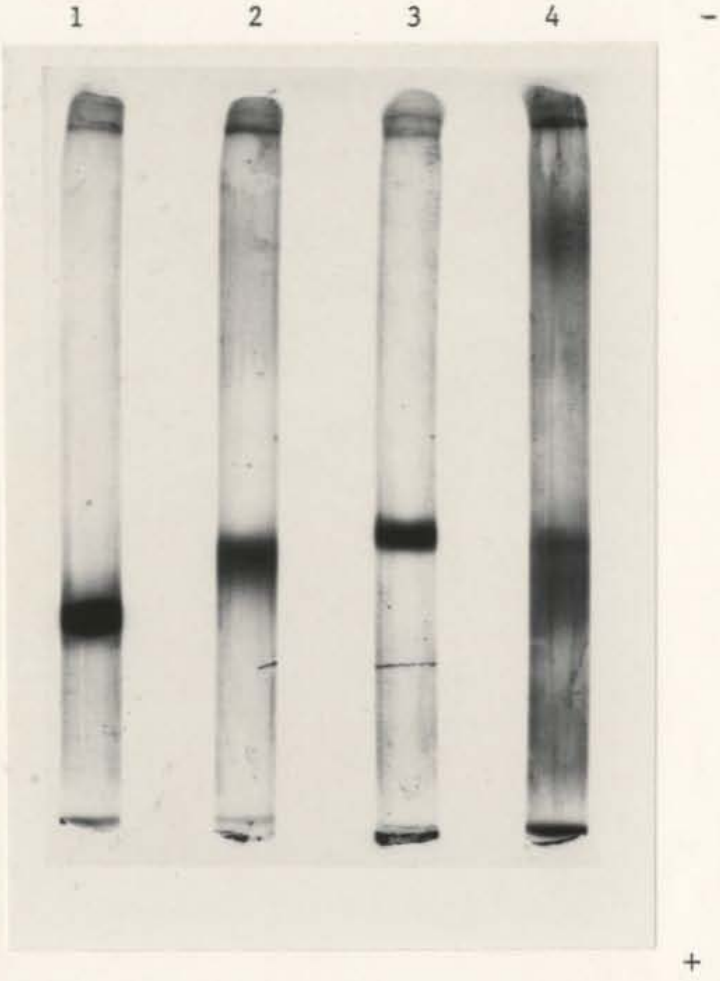
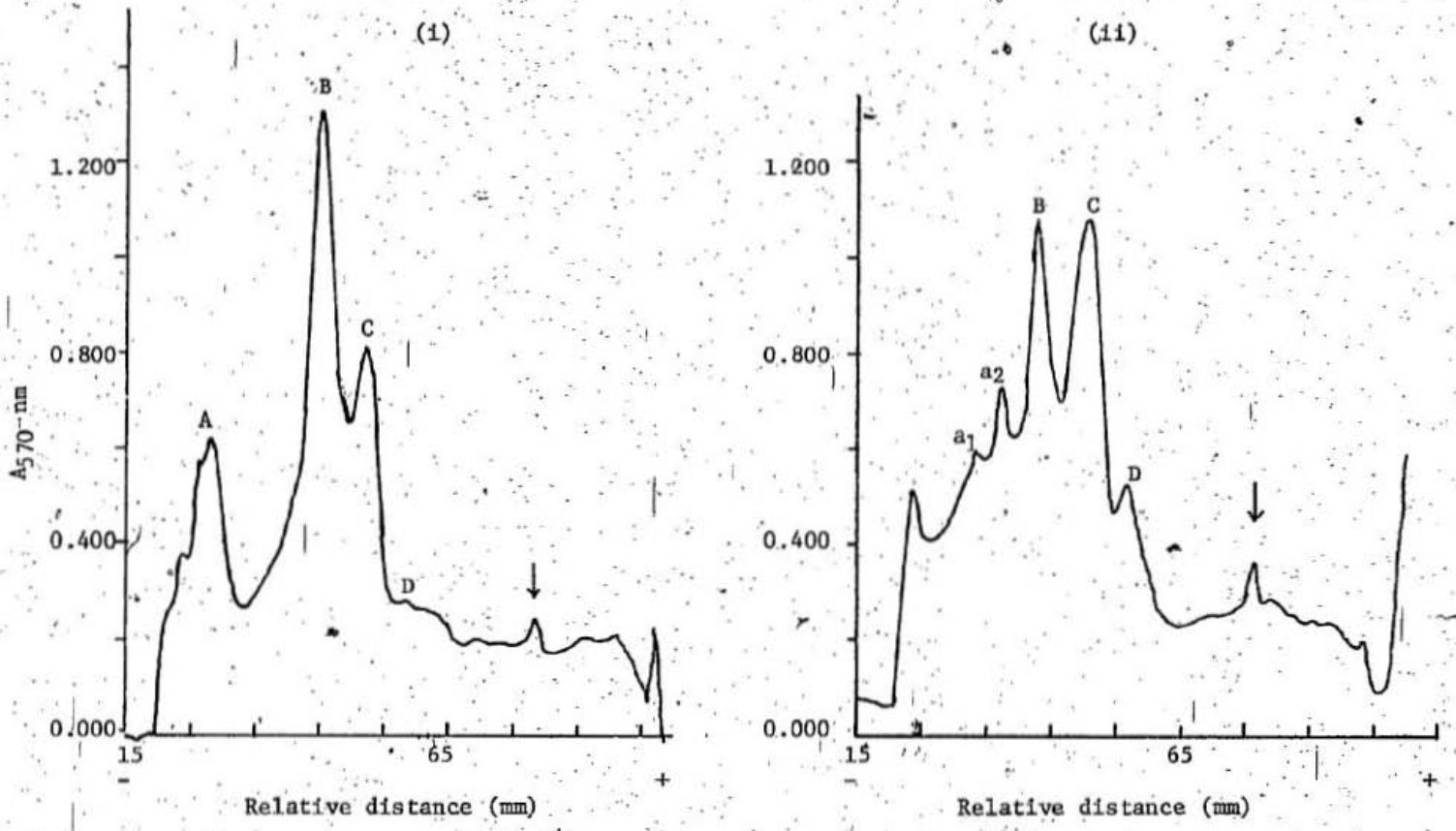

1
2
3
4
-
+

Figure 18. Gel scans of zymogen A and its active form.

Thirty µg of zymogen A purified by affinity chromatography and its enzyme form obtained by activating zymogen A at pH 2.0 were subjected to polyacrylamide gel electrophoresis according to the method of Davis (1964). The gels were stained in Coomassie blue. After destaining, the gels were scanned at 570 nm. (i) Zymogen A; (ii) protease A. The position of bromophenol blue is indicated by arrow.

The starting point of the scan was set at a point 15 mm from the start of the scan-length scale of the gel holder.


(i)
(ii)
1.200
0.800
0.400
0.000
A570 nm
A
B
C
D
a1
a2
15
65
−
+
Relative distance (mm)
Relative distance (mm)

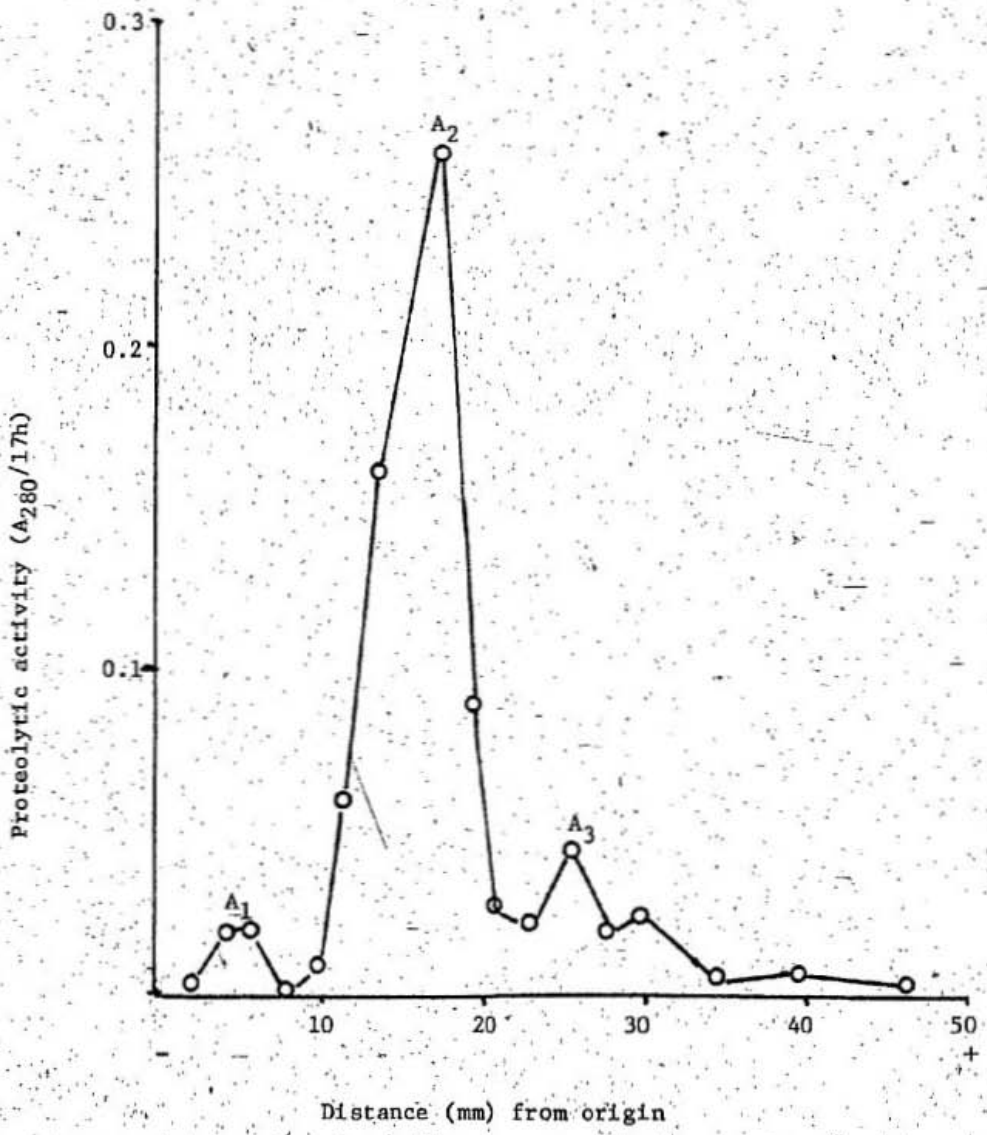
from 29% to 52% after activation. Peak D increased to 2.4% after activation from a faint band in zymogen A. The areas under various peaks were estimated by method of triangulation. These results strongly suggested that A and B are zymogens which are converted to faster moving bands. However, the activation seems to be partial and complex as the band B did not completely disappear on activation.

To determine which electrophoretic component of zymogen A is proteolytically active 50 µg of zymogen A purified by affinity chromatography was electrophoresed at pH 8.3 as described in Materials and Methods and the gel was sliced in 2 mm thickness. By incubating these slices with hemoglobin solution at pH 1.8 at 30°C for 17 hours highest activity was detected in a position corresponding to Rf 0.37 (peak A_2, Figure 19). There were minor activity peaks corresponding to Rf values of 0.10 and 0.55 (peaks A and A_3, respectively). However, based on the gel-scanning results shown in Figure 18 peak C is 29% of the total area whereas A_3 in Figure 19 represents only about 9% of the total pepsin activity. This may be due to instability of the active enzyme at high pH of the buffer used during electrophoresis. Two of the bands of protease A had mobilities close to those of two bands in calf chymosin (Rf 0.180 and 0.650). However, the mobility of one of the bands in calf chymosin (Rf 0.590) was also close to that of porcine pepsin which was homogeneous with Rf 0.570 (see Figure 17). The heterogeneity of the crystalline calf chymosin is probably because of the presence of more than one isoenzyme forms (Foltmann, 1960; Asato and Rand, 1977), and products of autodigestion.

(n) Molecular weight: Polyacrylamide gel electrophoresis in the

Figure 19. Detection of proteolytic activity on polyacrylamide gel

Fifty μg of zymogen A purified by affinity chromatography were subjected to polyacrylamide gel electrophoresis at pH 8.3 following the method of Davis (1964). Two mm thick slices from the gel were incubated with 1.5 ml hemoglobin solution at pH 1.8 at 30°C for 17 hours, and the product of hydrolysis determined spectrophotometrically as described in Materials and Methods.


0.3
0.2
0.1
A2
A1
A3
10
20
30
40
50
-
+
Proteolytic activity (A280/1h)
Distance (mm) from origin

presence of sodium dodecyl sulphate showed that zymogen A purified by affinity chromatography appeared as a single band (Figure 20) corresponding to a molecular weight of 33,800±1800 daltons based on an average of two estimations. This is higher than the molecular weight estimated by gel filtration (Table 7). Electrophoresis of protease A under the same conditions showed a broad diffuse band appearing at a position corresponding to molecular weight between 32,000 and 25,000 daltons. Purified zymogen C had a molecular weight of 44,000±2100 daltons and after activation the molecular weight of the main protein band was found to be 38,000 daltons. There was also a hazy band in the molecular weight range of 37,000-23,000 daltons.

(o) Amino acid compositions: The amino acid composition of the electrophoretic component of zymogen A with the highest proteolytic activity (peak A_2 of Figure 19) is shown in Table 8. This protein was isolated as follows: 480 µg of purified zymogen was electrophoresed on 12 analytical gels at pH 8.3 as described in Materials and Methods. A 3mm length from each gel was cut off at a position corresponding to Rf 0.37. The slices were combined into a test tube, macerated on a Vortex mixer with a spatula dipped into the gel. When the gels were broken to small pieces it was homogenised into a thin slurry with 3.0 ml of 0.1M NaCl using a polytron homogeniser (setting 4) for 3 minutes. The homogenate was centrifuged at 3,000xg for 30 minutes. The pellet was washed two more times with 3.0 ml of 0.1M NaCl. The washings were combined, dialysed against 2L water, pH adjusted with NH_4OH to 7.0. The dialysate was freeze-dried and subjected to amino acid analysis after 24 hour acid hydrolysis.

Figure 20. Polyacrylamide gel electrophoresis of the proteases and their zymogens in the presence of sodium dodecyl sulphate.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS PAGE) was performed as described in Materials and Methods. Zymogens A and C were purified by affinity chromatography and gel filtration respectively. A, zymogen A; B, protease A; C, zymogen C; D, protease C; and E, standard molecular weight markers as indicated by (a), bovine serum albumin (MW 65,000 daltons), (b) chicken egg albumin (MW 43,000 daltons), (c) ovine prolactin (MW 23,800 daltons), and (d), cytochrome C (MW 12,700 daltons).

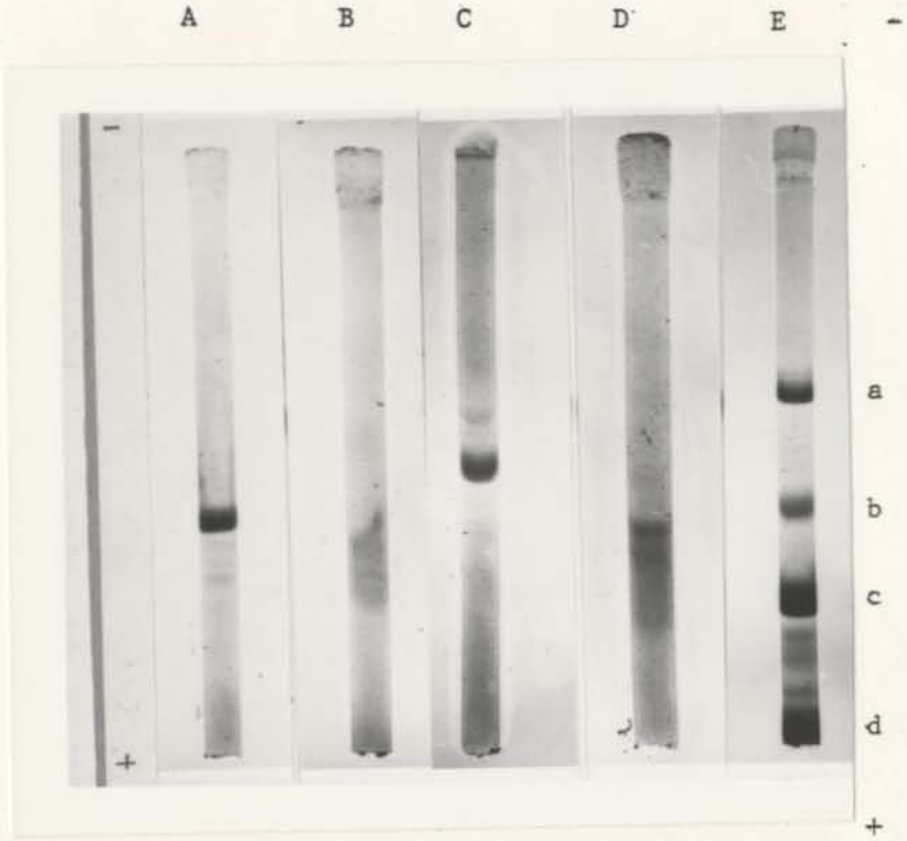

A
B
C
D
E
-
+
a
b
c
d
+

Table 7. Summary of the estimations of the molecular weights of zymogens A and C.

Method of analysis	Correlation coefficients of standard curve[1]	No. of trials	Estimated MW x 10^3
Zymogen A			Mean ± S.D.
Sephadex G-100	0.996	5	26,300±1540
HPLC	0.995	1	26,300
SDS PAGE	0.998	2	33,800±1800
Zymogen C			
Sephadex G-100	0.996	2	37,100±1075
SDS PAGE	0.998	2	44,000±2100

[1] Analysed by the method of least squares.

As shown in Table 8, there appears to be some similarity in the percentage compositions of aspartic acid, threonine, proline, glutamic acid, lysine, histidine and arginine for zymogen A and calf prochymosin. The percentages of the following amino acids tend to be different in the two zymogens: glycine, valine, leucine, tyrosine phenylalanine, serine, alanine and methionine plus its oxidized product(methionine sulphone). Isoleucine in prochymosin appeared to be twice as much as in zymogen A. Cysteine and cysteic acid together seemed higher in zymogen A than in prochymosin. Tryptophan was not determined in zymogens A and C.

There appeared to be some similarity between zymogen A and porcine pepsinogen as well. The percentages of serine, proline, valine, tyrosine and phenylalanine seemed similar. There was slight difference in the percentages of the following: threonine, aspartic acid, alanine, and tyrosine. However, there seemed to be a great difference in the percentages of the following: glutamic acid, glycine, cysteine(together with cysteic acid), methionine(together with its sulphone), isoleucine, leucine, lysine, histidine and arginine. Therefore, there seemed to be more similarity between zymogen A and prochymosin than between pepsinogen and prochymosin.

Zymogens A and C seemed to be similar with respect to aspartic acid, threonine, serine, glutamic acid, alanine, isoleucine, leucine, tyrosine, and phenylalanine. Percentages of proline, cysteine plus cysteic acid and valine appeared to be slightly different in the two zymogens. However, these two zymogens seemed greatly dissimilar with respect to the percentages of glycine, histidine, lysine and arginine.

Table 8. Comparison of Amino Acid Compositions[1]

	Zymogen A	Δ[5]	Prochymosin[2]	Zymogen C	Δ	Porcine Pepsinogen[3]	Δ
Cysteic Acid	0.8			Tr			
Methionine sulphone	0.3			0.0			
Aspartic acid	9.6	0.7	10.3	11.4	0.7	12.1	1.8
Threonine	6.3	0.1	6.2	6.2	0.1	7.2	1.0
Serine	12.3	2.6	9.7	13.2	3.5	12.7	3.0
Proline	4.7	0.3	4.4	6.7	2.3	5.2	1.8
Glutamic acid	11.8	0.6	11.2	11.0	0.2	7.7	3.5
Glycine	12.2	3.5	8.7	13.3	4.6	9.6	0.9
Alanine	6.7	2.0	4.7	6.6	1.9	5.2	0.9
Cysteine	2.0	0.1	1.9	1.9	0.0	1.7	0.2
Valine	5.9	1.3	7.2	4.4	2.8	6.3	0.9
Methionine	1.2	1.0	2.2	2.7	0.7	1.1	1.1
Isoleucine	3.3	2.6	5.9	3.9	2.0	6.9	1.0
Leucine	7.0	1.1	8.1	7.1	1.0	9.1	1.0
Tyrosine	4.1	1.5	5.6	4.9	0.7	4.7	0.9
Phenylalanine	4.1	0.9	5.0	4.2	0.8	4.1	0.9
Lysine	3.6	0.4	4.0	1.0	3.0	2.8	2.2
Histidine	1.9	0.3	1.6	0.5	1.1	0.8	0.8
Tryptophan	N.D.[4]		1.2	N.D.		1.7	
Arginine	2.3	0.1	2.2	1.1	1.1	1.1	1.1
Mean Δ		1.18			1.56		1.35

Notes:
1. Expressed as percentage of total.
2. Calculated from data reported by Ryle (1970).
3. Calculated from data reported by Foltmann (1970).
4. Not determined.
5. Δ = Difference from prochymosin.

VII. Preparation of cheddar cheese

Cheddar cheeses were prepared from 18L batches of milk with commercial calf rennet, Mucor miehei protease (Fromase), activated crude homogenate from harp seal gastric mucosa and activated DEAE peak A protease as milk coagulants. Some aspects of the cheese making process are summarised in Table 9. Although the initial pH of the pasteurised milk was almost the same for all the 4 lots of milk used, the time required for the pH to reach 6.4 after adding starter varied slightly. Although equal clotting units of all the coagulants were used setting time varied slightly, perhaps because of slight temperature difference of the milk. The pH of whey at the end of Cheddaring was between 5.4 and 5.5. There was an initial rapid drop in pH of the cheeses. One week after making the cheeses the pH was between 4.70 to 4.90. The pH of the cheeses were similar after 30 weeks of aging.

The yields of cheeses were very similar for all coagulants used (Table 10). Although there was slight difference in the total N values in whey, recovery of protein in the cheeses was similar (Table 11). There were slight differences in the fat contents of cheeses. It is difficult to judge the significance of these differences from data obtained from one lot of cheeses. However, data from a preliminary cheese-making trial(Appendix E) also appears to show that the yields are comparable between the cheeses made with calf rennet and SGP.

Table 9. Some parameters of cheese-making process

	Protease A	Crude SGP	Calf rennet	Fromase
pH of milk	6.60	6.50	6.50	6.50
pH at renneting	6.40	6.35	6.40	6.40
Temperature at renneting °C	30	31	31	30
Time between adding starter and coagulant (min)	40	50	45	50
Setting time (min)	25	20	20	22
pH at start of cheddaring	5.40	5.35	5.50	5.40
pH after one week	4.85	4.70	4.85	4.90
pH after thirty weeks	5.05	5.20	5.11	5.18

Table 10. Yields of cheddar cheeses and loss of nitrogen in whey

Enzyme used	Yield of cheese[1] %	Nitrogen in Whey[2]	
		Total %	Non protein %
Calf rennet	10.04	0.1091	0.0509
Protease A	10.08	0.1109	0.0515
Crude SGP	9.96	0.1227	0.0517
Fromase	10.00	0.1316	0.0510

1 After pressing and before vacuum packing cheeses, without adjustment for moisture differences.

2 Figures represent average of duplicate measurements.

Table 11. Proximate analyses of the Cheddar cheeses

Coagulant	Percent composition[1]		
	Fat	Moisture	Protein[2]
Calf rennet	30.79	38.41	25.19
Protease A	31.49	38.22	25.49
Crude SGP	30.58	38.26	25.23
Fromase	31.06	38.66	25.02

1 Numbers represent average of two determinations.

2 Obtained by multiplying Kjeldahl N values by a factor of 6.38.

Data from one analysis on one lot of cheeses.

VIII. Analyses of the cheeses

The sensory evaluation of the cheeses aged for 4 weeks and 30 weeks was performed by preference test by 30 panelists. The sensory scores were evaluated by analysis of variance (Larmond, 1977). As shown in Table 12 there was no significant difference in sensory preference scores between the 4-week-old cheeses made with calf rennet and protease A and between the cheeses made with calf rennet and SGP. However, the differences in scores between cheeses made with calf rennet and Fromase was significant at 5% level. After 30 weeks of aging the score for the cheese made with crude SGP was significantly higher than that for the cheese made with calf rennet at 5% level, as shown in Table 13. The sensory scores for the cheeses made with Fromase and protease A were not significantly different from that of the cheese made with SGP.

(a) Citrate-HCl extract: Figure 21 shows the absorption spectra of citrate-HCl extracts from 30-week-old cheeses. The wavelengths of maximum absorbance of citrate-HCl extract from cheeses were similar. Figure 22 shows the increase in absorbance of the citrate-HCl extracts with aging. It shows that initially the rate of ripening was faster for the cheeses made with crude SGP and Fromase up to 12 weeks when all the cheeses produced about the same amount of UV absorbing materials. After 30 weeks, however, the citrate-HCl extracts from the cheeses made with calf rennet and Fromase appeared to have higher absorbance than than the extracts from the cheeses made with SGP. This may indicate instability of SGP as shown by data in figures 10 and 13.

Table 12. Sensory evaluation of 4-week-old Cheddar cheeses[1]

Coagulant	Mean sensory score[2]
Calf rennet	6.37[a,b]
Protease A	6.87[a]
Crude SGP	5.77[a,b,c]
Fromase	5.50[c]

1. Evaluated by preference test by 30 panelists on a 9-point scale.
2. Standard error of mean = 0.26; least significant difference = 0.95.

Mean values followed by the same superscript are not significantly different at $p \leq 0.05$.

Table 13. Sensory evaluation 30-week-old Cheddar cheeses[1]

Coagulant	Mean sensory score[2]
Calf rennet	5.67^{b}
Protease A	$5.83^{a,b}$
Crude SGP	6.83^{a}
Fromase	$5.90^{a,b}$

1. Evaluated by preference test by 30 panelists on a 9-point scale.
2. Standard error of mean= 0.29; least significant difference = 1.07.

Mean values followed by the same superscripts are not different at $p \leq 0.05$.

Figure 21. Absorption spectra of citrate-HCl extract from 30 week old cheeses.

Citrate-HCl extracts of the cheeses were prepared by following the method of Vakaleris and Price (1959) and scanned in a Beckman DU8 spectrophotometer. a, Fromase; b, calf rennet; c, protease A; d, crude SGP

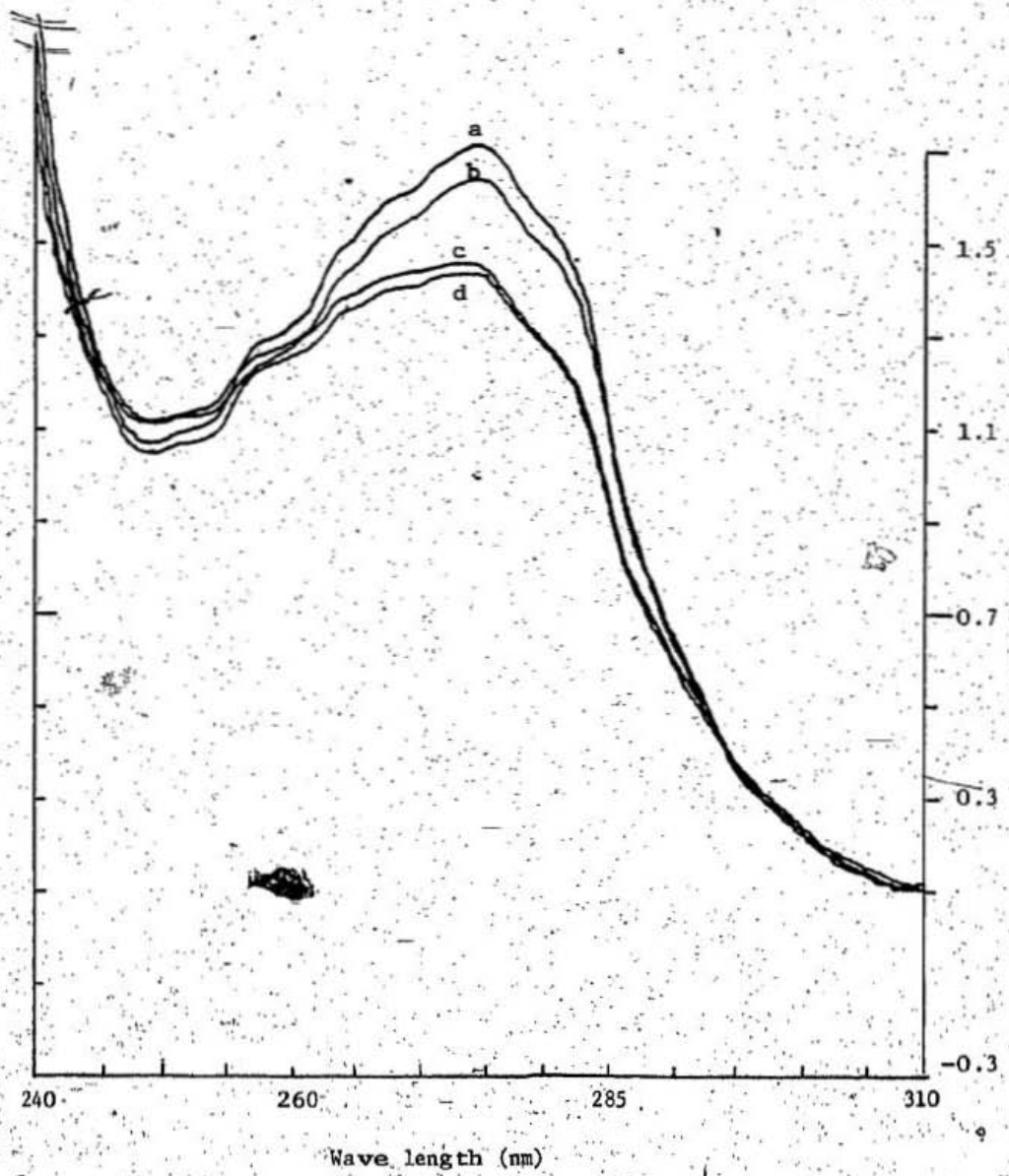
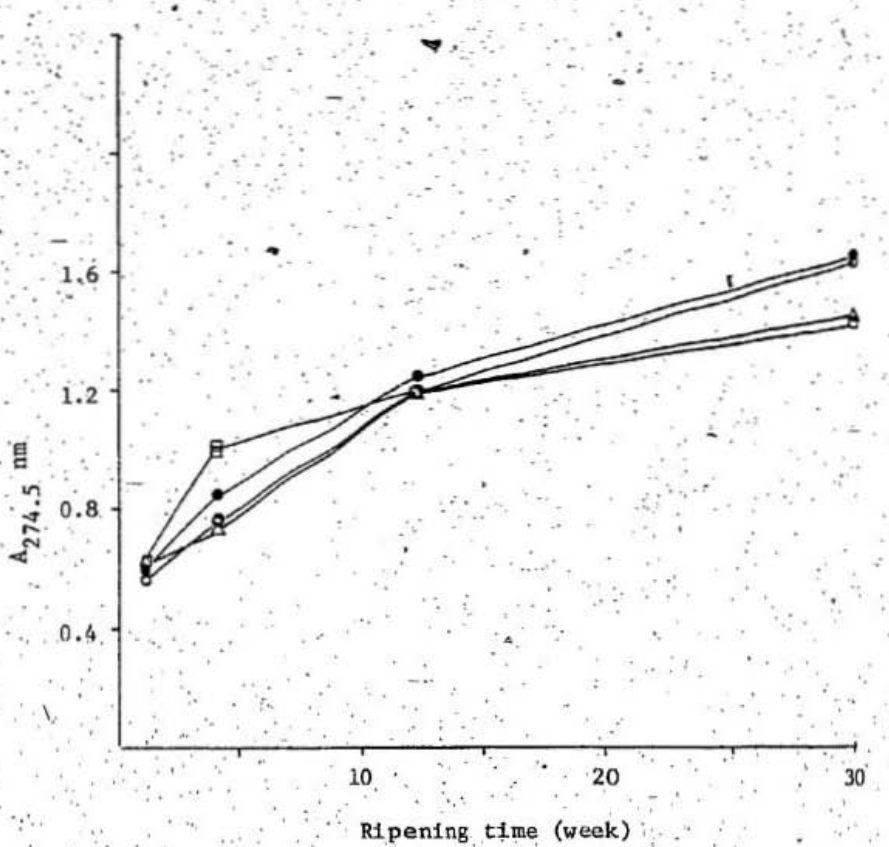

a
b
c
d
1.5
1.1
0.7
0.3
-0.3
Absorbance
240
260
285
310
Wave length (nm)

Figure 22. Spectrophotometric monitoring of protein degradation in ripening cheese.

Citrate-HCl extracts of cheeses at various periods of ripening were made following the method of Vakaleris and Price (1959) and absorbance measured at 274.5 mm. □, cheese made with crude SGP; △, cheese made with protease A; ○, cheese made with calf rennet; ●, cheese made with Fromase.

Data from one lot of cheese.


1.6
1.2
0.8
0.4
$A_{274.5\ nm}$
10
20
30
Ripening time (week)

The citrate-HCl extracts from 30 week old cheeses were analysed by polyacrylamide gel electrophoresis following the method of Davis (1964). The gels were stained and the mobilities of each component was determined using a Beckman DU8 gel scanner at 570 nm. The contribution of each peak to the total was calculated as the percentage of the total area (Table 14; photographs in Appendix L). There are similarities in the number of protein bands present in the extracts from the cheeses made with the SGP, protease A and calf rennet (bands a to p). The extract from Fromase cheese showed a lesser number of protein bands than was present in the extracts from the other three cheeses. There was considerable difference in the percentages of the fast-moving peptide designated q (Table 14) in the extracts from the four cheeses. The extract from Fromase cheese had the highest percentage of fast-moving bands whereas that from calf rennet cheese had the lowest percentage. The extract from crude SGP cheese had a percentage of fast-moving bands between these two values. This information may explain the higher sensory preference scores (Table 13) of the SGP cheese after 30 weeks of aging. However, the contribution of the peptides to the sensory quality of the cheeses would not be known until the peptides are isolated and subjected to sensory evaluation.

(b) Electrophoresis of whole cheese protein: Polyacrylamide gel electrophoresis of the whole cheeses was performed at pH 8.3 (Davis, 1964) at various stages of ripening. The protein in whole cheese samples was completely solubilized in citrate buffer containing urea as previously shown by Stanley and Emmons (1977). Figure 23 shows the pictures of gels superimposed on their scans from 30-week-old

Table 14. Percentage composition of proteins in citrate-HCl extracts from 30 week old cheeses

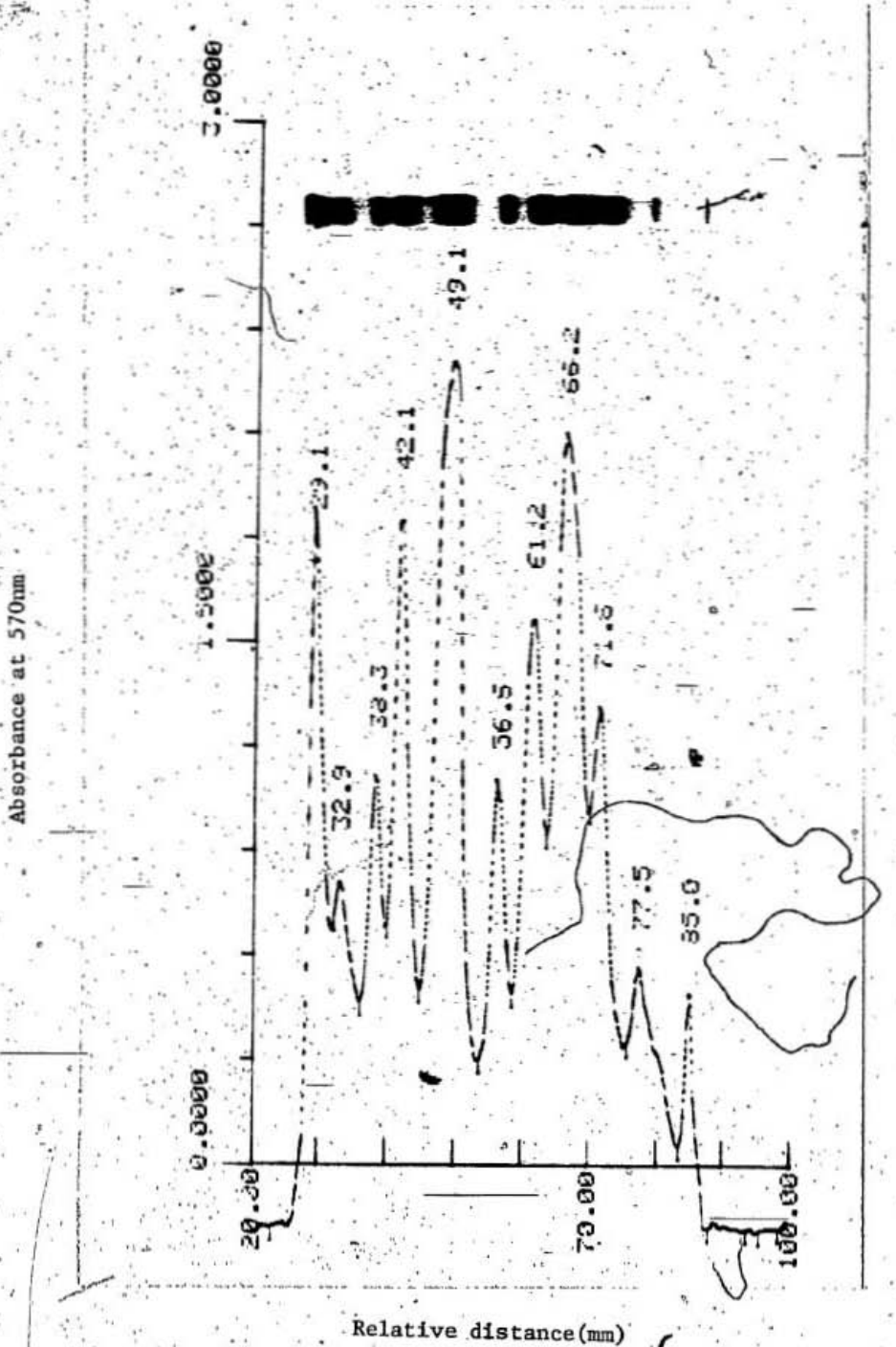
	Rf																
	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q
COAGULANT	0.040	.0750	0.121	0.156	0.210-0.233	0.256	0.313	0.340	0.371-0.378	0.402	0.469-0.479	0.520	0.589-0.608	0.643-0.669	0.780-0.821	0.882-0.923	0.954-0.978
Protease A	-	-	1.6	-	9.2	-	-	-	14.8	-	8.9	-	14.0	Trace	16.5	4.7	30.5
Crude SGP	2.6	-	-	-	17.2	-	-	-	6.0	-	6.8	-	8.0	2.6	11.9	4.3	40.7
Fromase	-	-	-	-	8.4	-	4.0	-	-	10.8	-	-	6.7	2.8	17.2	-	50.1
Calf Rennet	-	-	0.25	-	3.1	3.3	-	28.7	-	-	1.1	-	12.9	4.1	17.8	4.6	24.2
Whole Casein	-	4.7	-	2.1	4.9	-	-	32.2	-	-	-	5.4	-	43.0	7.7	-	-

Data from one analysis.

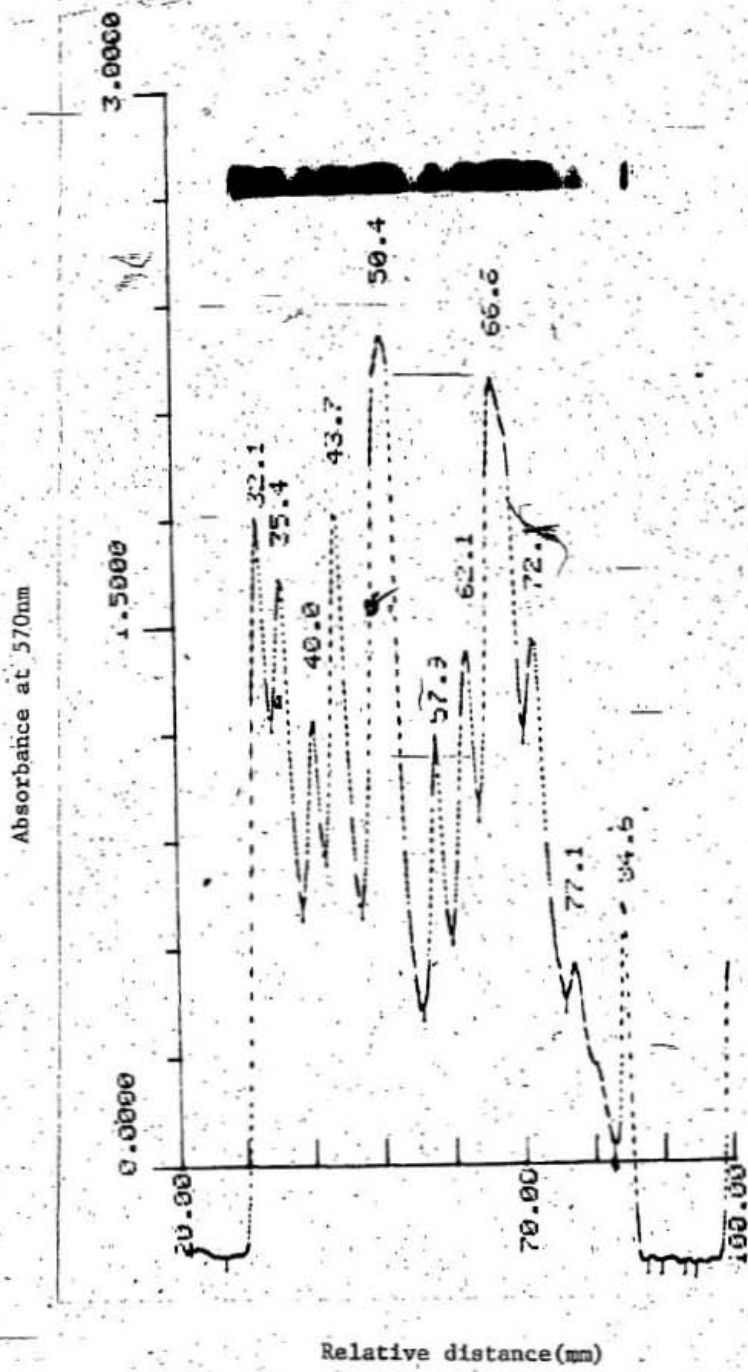
Figure 23. Polyacrylamide gel electrophoresis of whole cheese protein.

Samples from 30-week-old Cheddar cheeses were solubilized following the method of Stanley and Emmons (1977) and 10 µl aliquots electrophoresed according to the method of Davis (1964), stained, destained and scanned as described in the legend to Figure 23. Cheese made with (a) protease A, (b) crude SGP, (c) calf rennet, (d) Fromase.

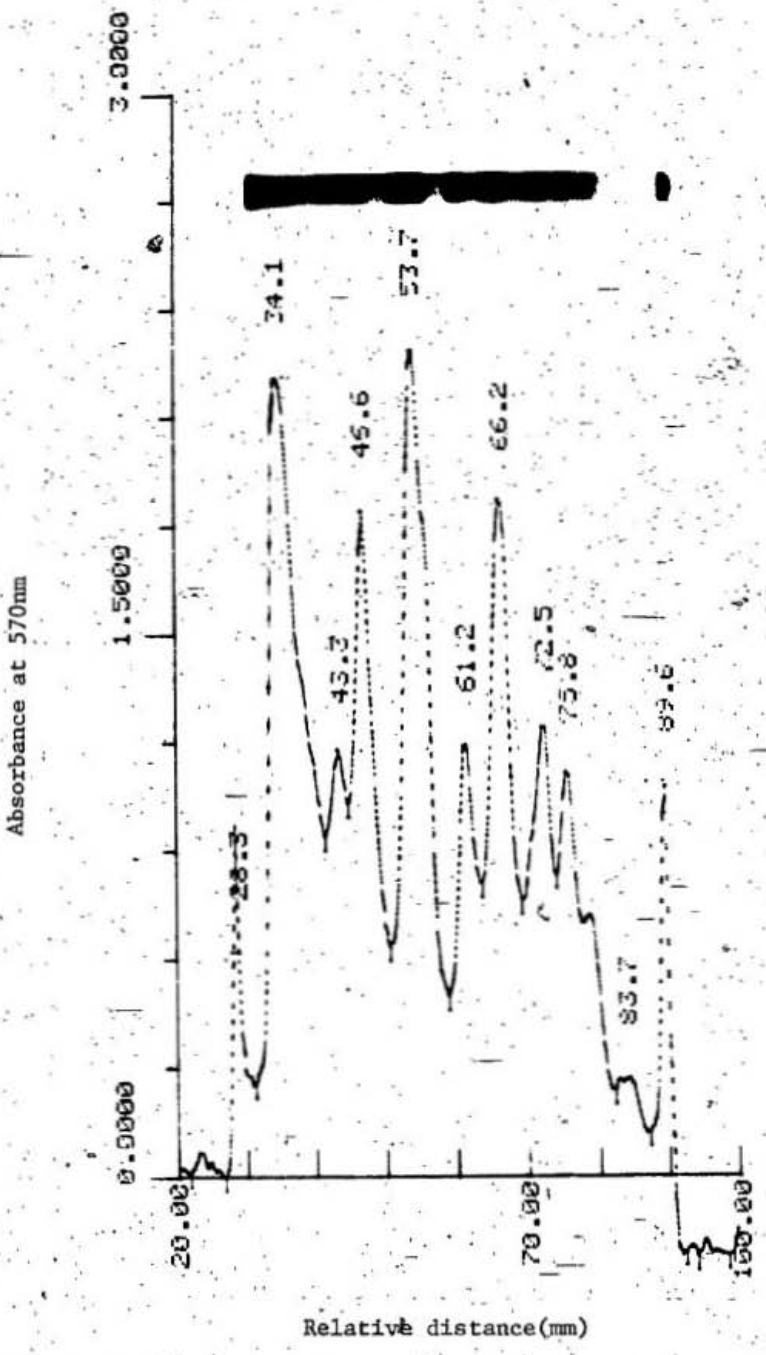
(a)


3.0000
1.5000
0.0000
Absorbance at 570nm
20.00
73.00
100.00
Relative distance(mm)
49.1
29.1
42.1
66.2
61.2
32.9
38.3
36.5
71.8
77.5
85.0

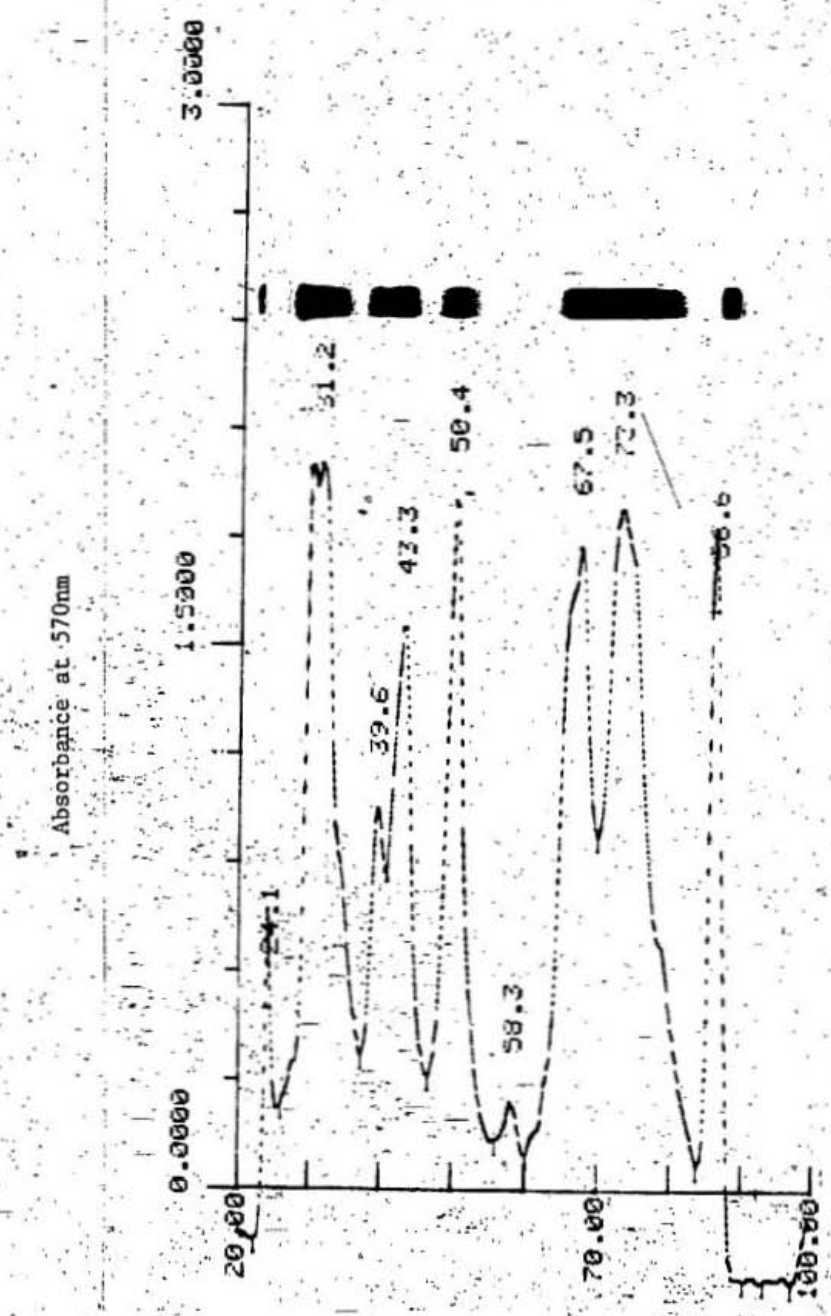
(b)


3.0000
1.5000
0.0000
Absorbance at 570nm
50.4
66.6
32.1
25.4
43.7
40.8
57.3
62.1
72.
77.1
84.5
20.00
70.00
100.00
Relative distance(mm)

(c)


3.0000
1.5000
0.0000
Absorbance at 570nm
20.00
70.00
100.00
Relative distance(mm)
24.1
28.3
43.3
45.6
53.7
61.2
66.2
72.5
75.8
83.7
89.6

(d)


3.0000
1.5000
0.0000
Absorbance at 570nm
31.2
39.6
43.3
50.4
58.3
67.5
72.3
20.00
70.00
100.00

cheeses. Whole casein was also electrophoresed as reference (Figure 24) each time the cheese samples were electrophoresed. The reproducibility of the Rf values of the various caseins and of their degradation products was good (0-4.7%) for the fast-moving components (E to M, Table 15) but poor for the relatively slow-moving components (A to D). The components of standard casein were identified on the basis of their relative mobility as shown by Stanley and Emmons (1977). Para κ-casein component (Rf=0.07) was not observed in one-week-old cheese samples. However, a band was present at the starting point of the small-pore gel. This may include para-κ-casein plus the degradation product of other casein components with overlapping mobility because the percentage of this band was higher than that of κ-casein in the whole casein gel. In the 30-week-old cheeses there were two bands in the position of para-κ-casein in the cheeses made with protease A and SGP whereas in the case of cheeses made with calf rennet and Fromase the two bands were merged. These two components were estimated together and recorded as para-κ-casein. Therefore what has been reported here as para-κ-casein is perhaps the sum of para-κ-casein and some other peptides. In all the cheeses the percentages of para κ-casein, Ts-, and γ-casein increased after 30 weeks of aging. However, it should be pointed out here that since changes in percentage composition estimated this way are only relative, an increase in the percentage of one component may also suggest that the component in question has changed slowly or not at all, while the other components have diminished faster (Stanley and Emmons, 1977).

The most remarkable differences are in the major casein components α_{s1} and β. The α_{s1}-casein was much more extensively degraded in the

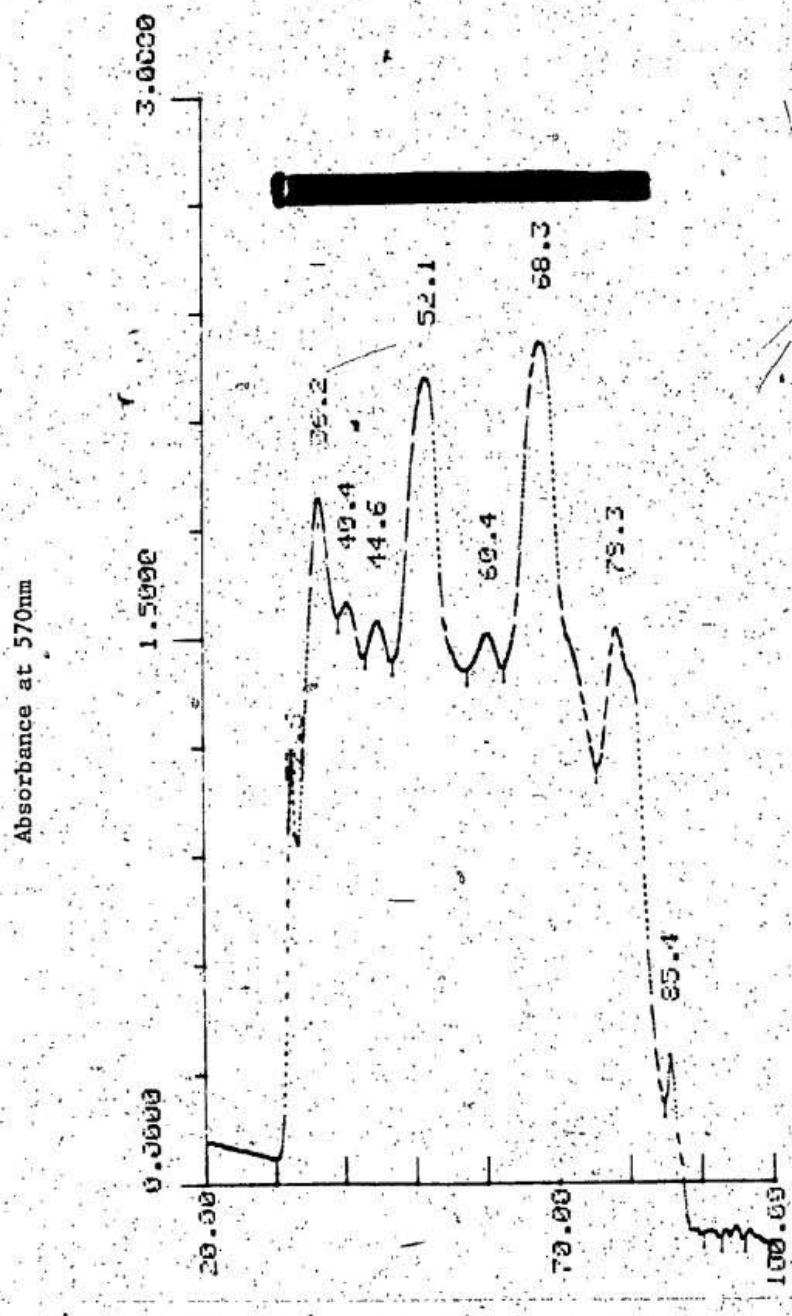
Figure 24. Polyacrylamide gel electrophoresis of whole casein.

Acid casein prepared following the method of Fox and Guiney (1976). Forty mg of freeze-dried casein was dissolved in 10 ml of 6M urea containing 0.1%, potassium citrate (Stanley and Emmons, 1977) and 15 µl used for electrophoresis following the method of Davis (1964). Other conditions described in legend to Figure 23.

Identification of casein components

	Relative distance[1] (mm)
κ-casein	36.2
γ-casein	40.4
T_S-casein	44.6
β-casein	52.1
Minor α_S casein	60.4
α_{S1}-casein	68.3
Minor α_S casein	78.3

1. Identifying numbers appearing above each peak represents its distance in millimeters from the start of the scan length scale of the gel holder. The starting point of the scan was set at a point 20 mm from the start of the scale.


3.0000
1.5000
0.0000
Absorbance at 570nm
35.2
52.1
68.2
40.4
44.6
60.4
75.3
85.4
20.00
70.00
100.00
Relative distance(mm)

30-week old calf rennet cheese (Table 15, Figure 25a, b) than in the other three cheeses. In the Fromase cheese the degradation of α_{s1} casein was less extensive than in calf rennet cheese but β-casein underwent the highest degradation. In the cheeses made with protease A and SGP, the percentages of residual α_{s1}-casein were higher than in calf rennet cheese but the percentages of residual β-casein were comparable to that in the calf rennet cheese.

Some protein bands with mobilities different from those of standard casein components appeared with aging of the cheeses in positions E, G, H, J, K, L, and M (Table 15). Change in the percentage compositions of most of these proteins in the seal protease cheeses were similar to those of the calf rennet cheese after 30 weeks of aging (Table 15).

(c) Gel filtration of cheese extracts: Samples from 30 week old cheeses were dissolved in 0.1M tris citrate buffer pH 8.6 containing 6M urea, 1 mM EDTA and 1 mM DTT according to the method of Foster and Green (1974) and chromatographed on a Sephadex G-100 column to estimate the sizes of proteins and the extent of their degradation. To estimate the percentage compositions of the proteins a triangulation method was followed for calculation of peak areas. Since the peaks were not resolved completely the base-line was connected to the lowest point between the unresolved peaks. Figure 26 shows the elution patterns. The approximate molecular weights of the proteins and their percentage compositions are listed in Table 16. The percentages of low-molecular weight proteins (less than 12,700 daltons) in the cheeses made with the seal proteases were less than that in the Fromase cheese but higher than

Table 15. Degradation of Caseins in Cheese with Ripening

	Percentage Composition												
	A	B	C	D	E	F	G	H	I	J	K	L	M
	para-κ casein	γ-casein	Ts-casein	β-casein		α_{s0}-casein		α_{s1}-casein					
	(.068-.079)	(.156-.189)	(.266-.263)	(.344-.390)	(.489-.505)	(.527)	(.573-.578)	(.638-.668)	(.671-.676)	(.747-.763)	(.805-.820)	(.860-.890)	(.980)
COAGULANT													
Calf rennet													
1 week	15.3	0.9	1.6	37.2	0.7	-	-	24.0	20.3	Trace	-	-	-
4 weeks	19.0	0.4	5.2	33.3	2.4	-	1.2	12.9	21.8	-	-	-	-
30 weeks	19.6	4.6	9.8	19.2	5.3	-	16.8	9.4	-	9.4	2.0	1.8	2.4
Protease A													
1 week	12.2	3.4	3.7	32.0	2.2	-	-	39.9	-	6.6	-	-	-
4 weeks	16.1	3.1	4.2	32.2	2.4	-	-	40.4	-	Trace	-	-	-
30 weeks	11.3	6.2	9.7	21.0	4.5	-	10.6	23.1	-	9.2	-	3.9	1.0
Crude SGP													
1 week	9.9	2.3	3.4	38.0	1.9	-	-	43.0	-	-	-	-	-
4 weeks	18.0	2.3	2.6	30.9	4.5	-	-	38.3	-	3.3	-	-	-
30 weeks	15.2	4.7	10.1	20.8	3.8	-	8.0	22.9	-	9.8	-	1.4	1.2
Fromase													
1 week	16.4	3.4	4.5	30.2	2.1	-	-	36.7	-	-	6.2	0.6	-
4 weeks	21.0	2.9	3.5	25.8	2.8	-	-	17.4	11.9	7.5	6.5	-	-
30 weeks	13.6	4.5	11.4	12.6	0.6	-	-	18.8	-	31.1	Trace	-	7.5

Note: The numbers in parentheses represent the range of Rf. values of the components of cheese proteins and those of standard casein calculated from 3 determinations.

Figure 25. Degradation of major casein components during ripening of Cheddar cheese.

Samples from 1 week, 4 week and 30 week old Cheddar cheeses were subjected to polyacrylamide gel electrophoresis as described in materials and methods and the gels scanned in a Beckman DU8 spectrophotometer equipped with a gel scanner capable of area calculation. The figures show changes in each casein component as a percentage of total casein residues in cheese with aging. (a) and (b) α_{s_1}-casein (——), β-casein (- - -), (c) para-K-casein (——), T_s-casein (- - -), (d) γ-casein. ○, cheese made with calf rennet; ●, cheese made with Fromase; △, cheese made with protease A, □, cheese made with crude SGP. The data is for 1 analysis for 1 lot of cheese.

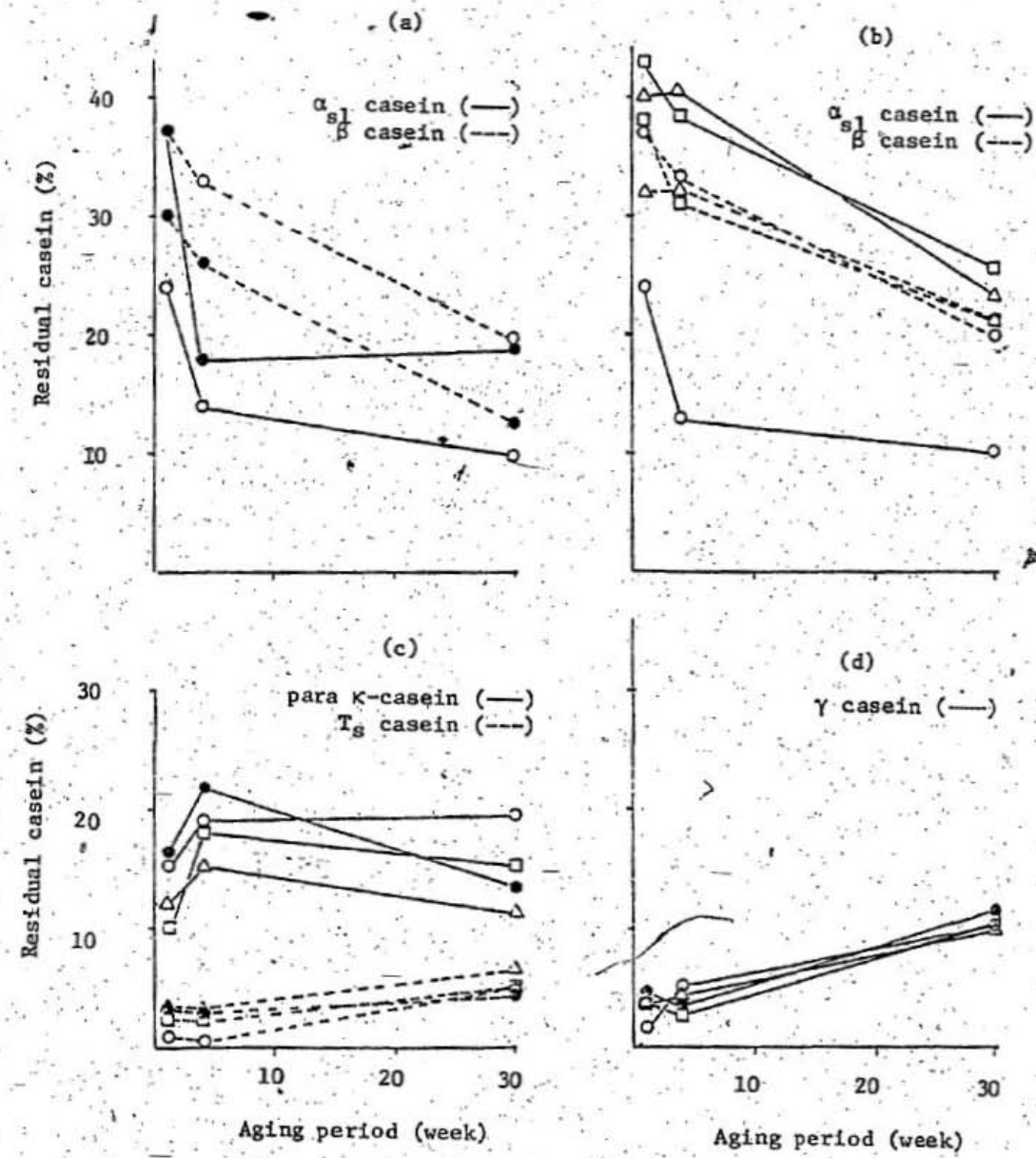

(a)
α_{s1} casein (——)
β casein (---)
Residual casein (%)
40
30
20
10
(b)
α_{s1} casein (——)
β casein (---)
(c)
para κ-casein (——)
T_s casein (---)
Residual casein (%)
30
20
10
10
20
30
Aging period (week)
(d)
γ casein (——)
10
20
30
Aging period (week)

Figure 26. Separation of cheese proteins on Sephadex G-100 column. Samples from 30-week-old cheeses were dissolved in buffer as described by Foster and Green (1974) and 5.6 ml solution applied to a 1.6 x 88.5 cm column of sephadex G-100. Void volume of the column,52 ml. Flow rate 6ml/h, fraction size, 2.0 ml.

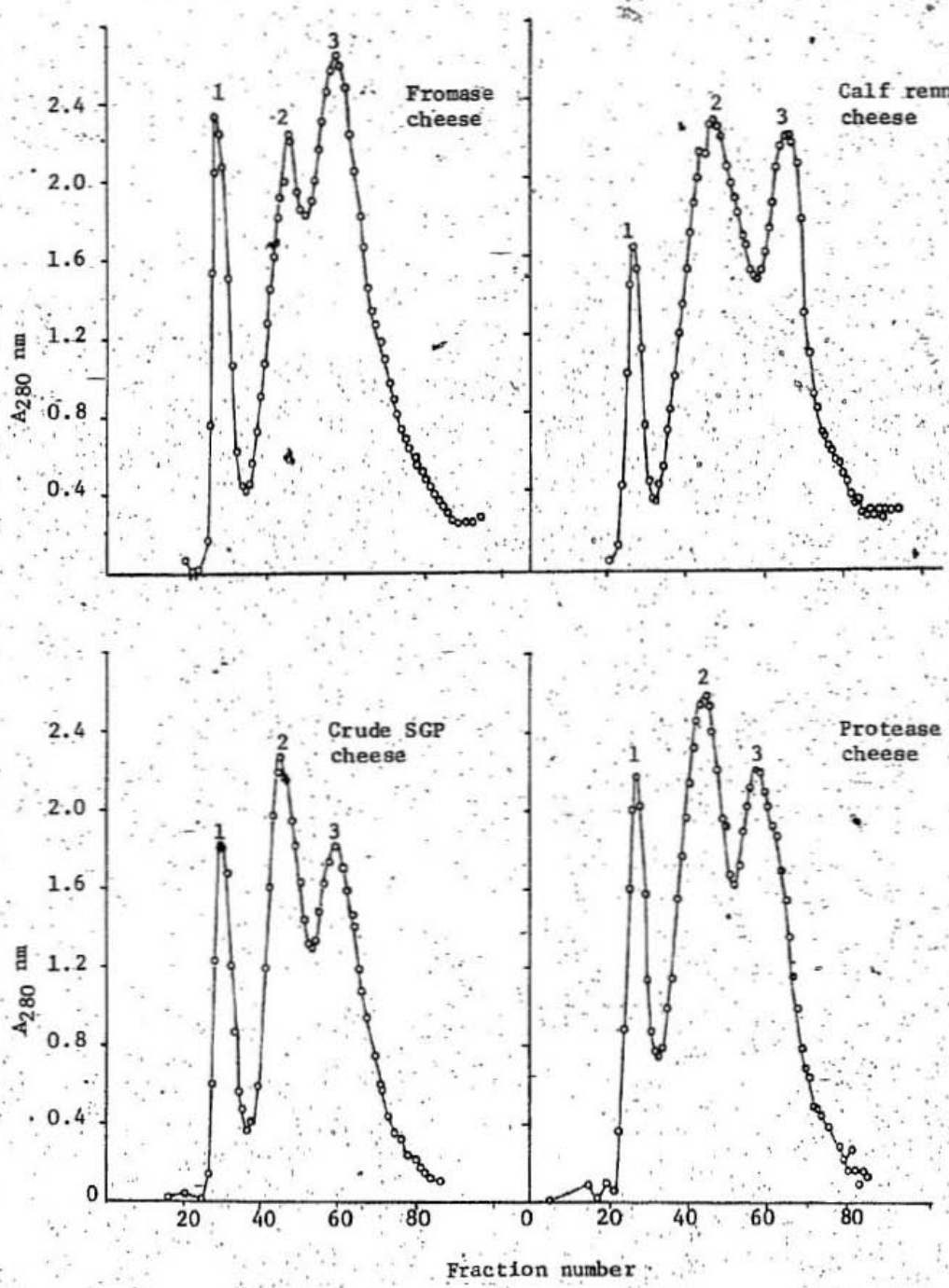

Fromase cheese
Calf rennet cheese
Crude SGP cheese
Protease A cheese
A280 nm
Fraction number
1
2
3
0.4
0.8
1.2
1.6
2.0
2.4
0
20
40
60
80

Table 16. Protein degradation patterns in 30-week-old Cheddar cheeses estimated by gel filtration

Coagulant	Protein composition(%)[1]		
	Peak 1 (70,000 daltons)	Peak 2 (30,000 daltons)	Peak 3 (<12,700 daltons)
Fromase	12.6	33.6	53.7
Protease A	14.3	46.4	39.0
Crude SGP	15.4	45.5	39.1
Calf rennet	9.6	58.1	32.3

[1] Estimated by triangulation method. Since the peaks were not resolved calculation was done by connecting the base line to the lowest point between two unresolved peaks.

that in calf rennet cheese. This observation supports that summarised in Table 14 which shows that the percentages of the fast-moving peptide q in the extract from SGP cheeses were between those of the extracts from Fromase and calf rennet cheese. Although the molecular weights of the casein components are between 18,000 and 23,000 daltons, a considerable amount of protein in the cheeses had molecular weight higher than those of caseins indicating the aggregation of the proteins even in the presence of 6M urea. Foster and Green (1974) also made similar observations. These authors also observed that in the fresh curd the protein peaks of molecular weights between 11,000 and 22,000 daltons were relatively small but increased in size during aging.

(d) Amino acid analysis of cheeses: Citrate-HCl extracts from 1 week and 30-week-old cheeses were analysed for both free and total (i.e. hydrolysate) amino acids. Results are shown in Figure 27 A and B. Amino acid composition of the citrate-HCl extract of old "Forfar" Cheddar cheese - a very popular brand of Cheddar cheese is also included for comparison (Figure 28).

The free amino acid content of 1-week-old cheeses were about 4 to 6 μmoles/g cheese (see Appendix F). The compositions of the total amino acids were also similar for the extracts from all the one-week-old cheeses (Appendix H). After 30 weeks of aging free amino acid levels rose significantly (Appendix F) to 33-39 μmoles/g cheese. The levels of free amino acids in the two SGP cheeses were somewhat lower than those of calf rennet and much lower than Fromase cheese (Appendix G, J and Figure 27). Cheeses made with protease A and crude SGP showed lower levels of phenylalanine and higher levels of cysteic acid and

Figure 27. Amino acid compositions of the Citrate-HCl extracts from Cheddar Cheeses.

Free amino acids were determined on the clear supernatant obtained by treating the Citrate-HCl extracts with 4 volumes of 20% sulphosalicylic acid. The total amino acids were determined by hydrolysing the Citrate-HCl extracts in 6N HCl for 24h at 110°C. Amino acids have been numbered as follows:

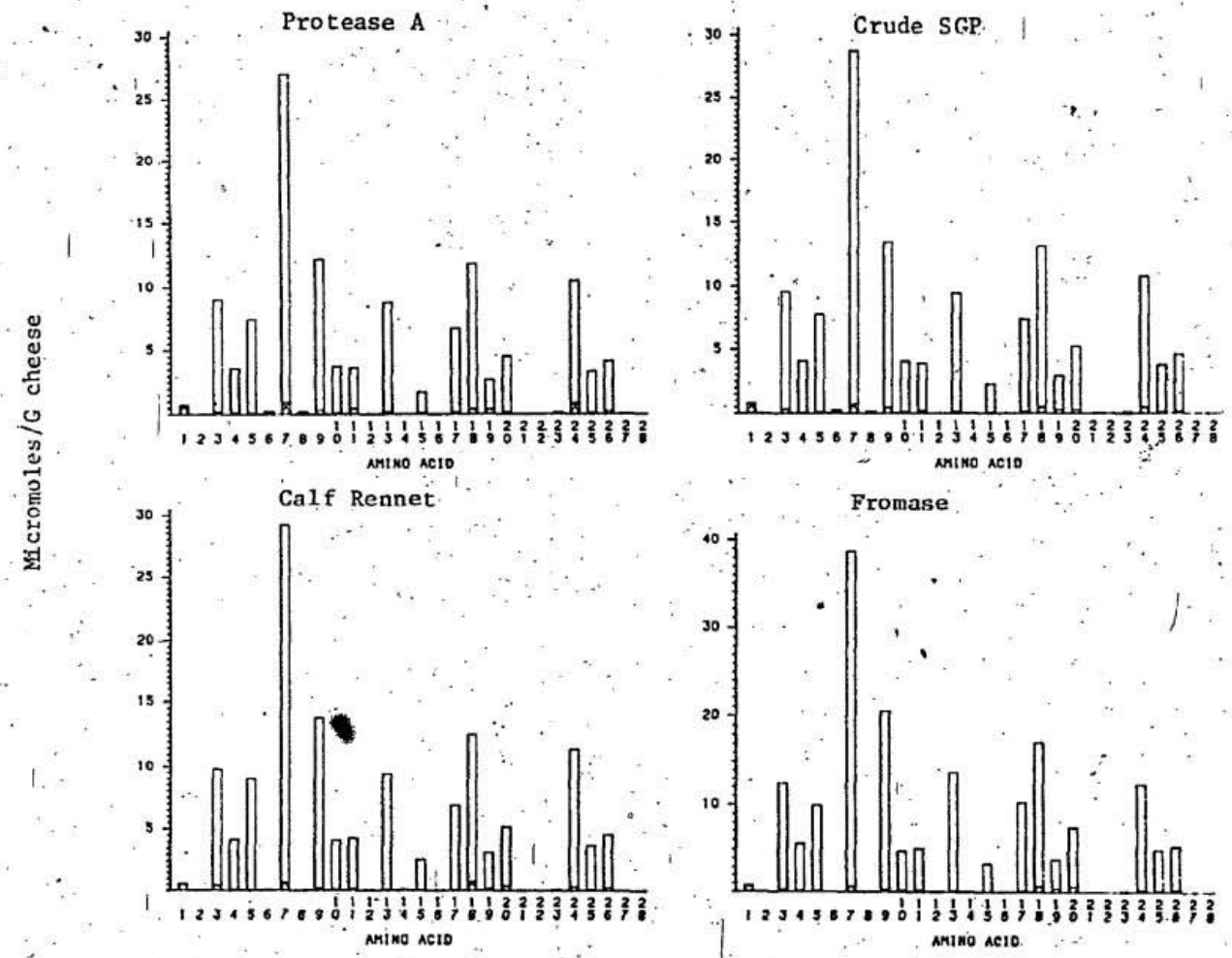
1. Cysteic acid
2. Glycerophosphoethanolamine
3. Aspartic acid
4. Threonine
5. Serine
6. Asparagine
7. Glutamic acid
8. Glutamine
9. Proline
10. Glycine
11. Alanine
12. Citrulline
13. Valine
14. Half cystine
15. Methionine
16. Cystathionine
17. Isoleucine
18. Leucine
19. Tyrosine
20. Phenylalanine
21. γ-Amino butyric acid
22. Tryptophan
23. Ornithine
24. Lysine
25. Histidine
26. Arginine
27. Taurine
28. β-Alanine

Legends: Cross-hatched bar, free amino acid;

Open bar, total amino acids.

(a) One week old cheeses; (b) Thirty week old cheeses

(a)


Protease A
Crude SGP
Calf Rennet
Fromase
Micromoles/G cheese
AMINO ACID

(b)

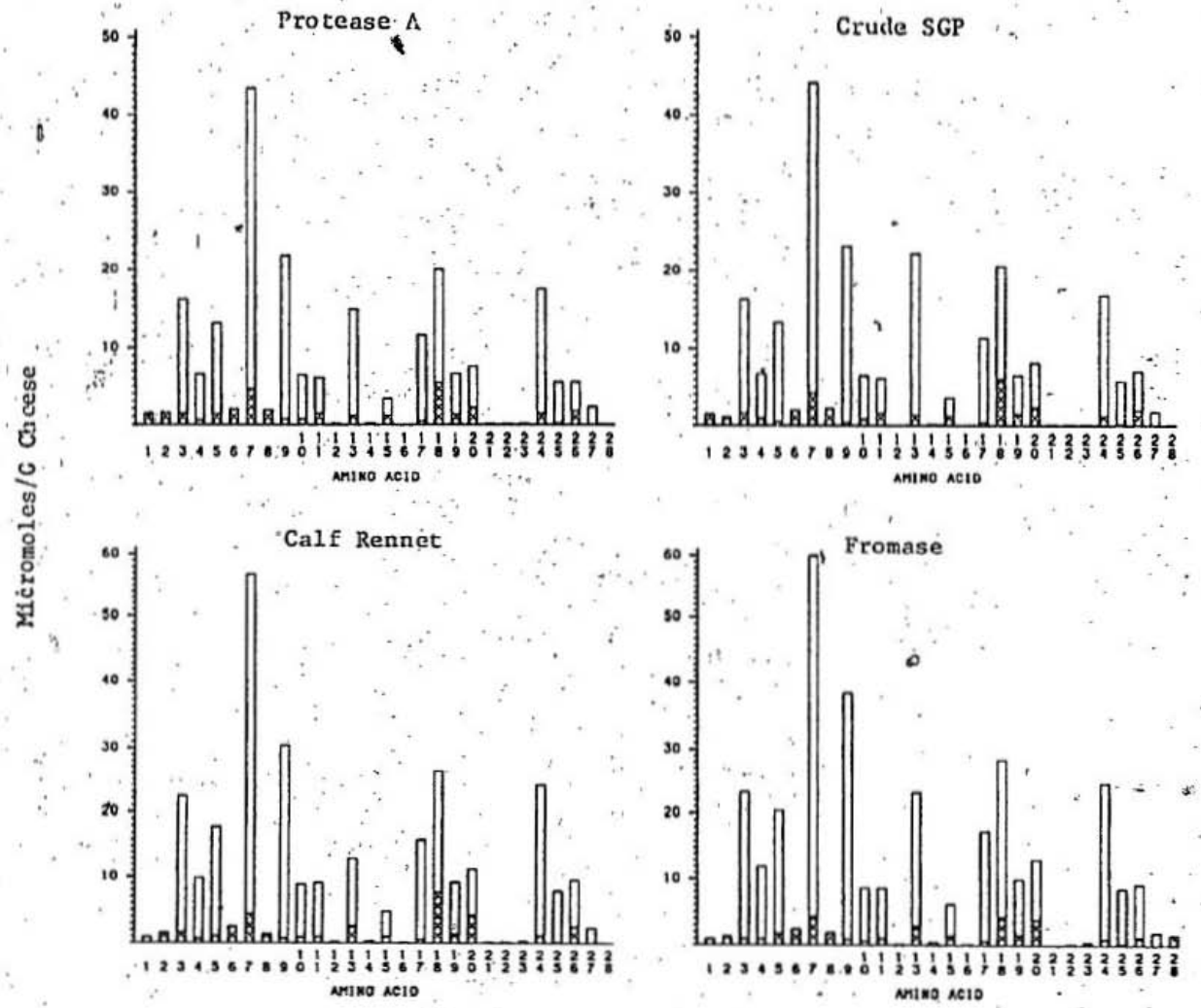
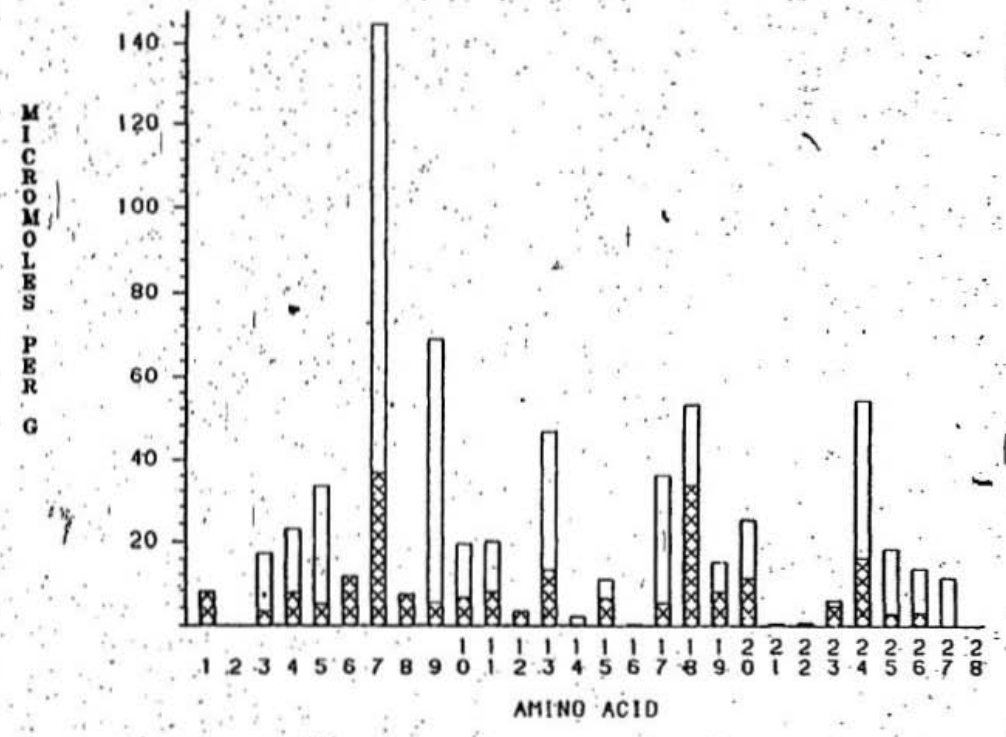

Protease A
Crude SGP
Calf Rennet
Fromase
Micromoles/G Cheese
AMINO ACID

Figure 28. Amino acid composition of the citrate-HCl extract from old "Forfar" Cheddar cheese.

Legend same as in Figure 27.


MICROMOLES PER G
140
120
100
80
60
40
20
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
AMINO ACID

glutamine than in calf rennet cheese (see Appendix G). The level of threonine was higher in crude SGP cheeses than in the cheese made with protease A. Whereas the levels of glycerophosphoethanolamine, serine, and proline were lower. Cheese made with Fromase showed higher levels of threonine, serine, glutamine, valine, methionine and ornithine while the levels of aspartic acid and leucine and arginine were lower compared to calf rennet cheese. Except for valine which had a higher percentage in the extract from 30 week old crude SGP cheese than in the other three cheeses, the percentage compositions of the total amino acids for all the four cheeses were very similar (see Appendix I).

"Forfar" is considered an outstanding Cheddar cheese, which is manufactured in Ontario. No details were available from the retailer about the cheese except that it was aged for 1-2 years and was prepared with calf rennet with no cheese color or other additives added. This cheese is presumably manufactured from raw milk. None of the cheeses prepared in this study had any added color but there was significant color similarity between the 30-week-old SGP cheeses and the "Forfar" cheese. "Forfar" and the two SGP cheeses had a yellow tinge which appeared to be absent in the Fromase and calf rennet cheeses.

Free amino acid levels in the citrate-HCl extract from "Forfar" cheese was much higher than any of the aged cheeses of this study as shown in Figure 28. Percentages of free aspartic acid, arginine and phenylalanine are particularly lower and the percentages of free cysteic acid, glutamic acid, citruline, isoleucine, ornithine, lysine and histidine are higher in "Forfar" than in the calf rennet cheese. The percentages of total aspartic acid and arginine are lower and the percentages of total valine, ornithine and taurine are higher in the

"Forfar" than in the calf rennet cheese. High levels of amino acids or differences in relative proportions do not seem to explain the observed sensory quality of the four cheeses made in this laboratory. Likewise, these higher levels of amino acids in "Forfar" cheese than in the calf rennet cheese may not be entirely responsible for its superior quality. "Forfar" cheese is believed to be manufactured with raw milk. Other manufacturing conditions such as the type of starter culture and the type of rennet used in the "Forfar" are not known. These are also very important in the development of flavor (see (Fryer, 1969 for a review).

GENERAL DISCUSSION AND CONCLUSION

"Pepsinogens" were isolated from the stomach mucosae of young and adult harp seals (Pagophilus groenlandicus). The two major pepsinogens were purified, activated and characterised with emphasis on the chymosin-like properties and use as a rennet substitute.

I. Physical properties of SGP and other gastric proteases: The crude estract from young and adult harp seal gastric mucosa separated into four active components by ion exchange chromatography on DEAE-Sephadex A-50 column (Figure 2) or isoelectric focusing (Figure 15). Based on the relative peak sizes pepsinogens A and C from ion exchange chromatography comprised the majority of the pepsin activity of the crude extract; components B and C' occurred in relatively small proportions.

Occurrence of multiple isoenzymes had been reported in stomachs of other species as well. Human stomach secretes 7 pepsinogens (Etherington and Taylor, 1970) and monkey stomach secretes 5 pepsinogens (Kageyama ang Takahashi, 1976). Chicken stomach secretes 4 (Donta and Van Vunakis, 1970) or 5 pepsinogens (Green and Llewellin, 1973). Stomachs of hog (Fruton, 1971), cow (Meitner and Kassel, 1971) and dogfish (Bar-Eli and Merret, 1970) secrete 4 pepsinogens. Castle and Wheelcock (1971) showed that calf secretes 2 prochymosins. Gastricsin, another proteolytic enzyme, has been reported to occur in stomachs of several species (Tang, 1970; Ward et al., 1981; Ryle, 1960; Chiang and Ponce, 1981). However, multiple gastricsins have been reported only in the fish Merluccius gayi (Chiang and Ponce, 1981).

The electrophoretic mobility of zymogen A determined by the method

of Davis (1964) was much slower than that of zymogen C. The main protein band of protease A also showed much slower mobility than those of protease C, porcine pepsin and also those of the major components of calf chymosin. However, both chymosin and protease A had a slow moving band.

It is interesting to note that protease A is electrophoretically faster than its precursor and this observation is similar to that of Asato and Rand (1977) who noted that all chymosins from calf stomach were electrophoretically faster than their respective precursors. On the other hand, chicken pepsins were found to move slower than the corresponding pepsinogens (Donta and Van Vunakis, 1970), similar to the pepsin/pepsinogen C of harp seal observed in this study. Therefore the relative electrophoretic mobilities of zymogen and enzyme also suggest that protease A is a chymosin-like enzyme.

Isoelectric focusing of the crude enzyme from the gastric mucosa of a two year old harp seal revealed that there are 4 isoenzymes M, N, O, and P as shown in Figure 15. The isoenzyme M which had a higher CU:PU than the other three had an isoelectric point of 4.90, similar to that of calf chymosin with a pI of 4.70 (Righetti et al., 1977). The isoelectric points of the other three isoenzymes were 4.31, 3.82, and 3.40. These values are higher than the reported isoelectric points of bovine and porcine pepsins but similar to those of chicken pepsin (Righetti et al., 1977).

The molecular weight of zymogen A estimated by gel filtration was 26,300 daltons; a similar molecular weight was estimated by HPLC. This is much lower than those of most pepsinogens and also lower than that of calf prochymosin. However, the molecular weight of zymogen A

estimated by SDS polyacrylamide gel electrophoresis was close to that of calf prochymosin. Zymogen C had an estimated molecular weight of 37,100 daltons by gel filtration and 44,000 daltons by SDS polyacrylamide gel electrophoresis (Table 7). Similar discrepancies in the molecular weights of porcine pepsin estimated by two different methods were reported before (Dieu, 1956; Edelhoch, 1957). Chiang and Ponce (1981) reported the molecular weights of gastricsinogens I and II from M. gayi to be 27,000 and 28,000 daltons by gel filtration and 23,300 and 33,300 daltons, respectively, by SDS PAGE. Actual molecular weight of zymogen A would be known only after complete elucidation of its primary structure.

II. Catalytic properties of SGP and other gastric proteases: All the four isoenzymes of harp seal gastric proteases showed milk-clotting and hemoglobin-hydrolytic activities. However, the milk-clotting activity of the isoenzymes per unit of their peptic activity called CU:PU differed for the isoenzymes. The CU:PU ratio for protease A was higher than that of the other major protease C but was similar to that for chymosins from calf (Green, 1972), kid and lamb (Anifantakis and Green, 1980). Piglet "chymosin" was reported to have a CU:PU higher than that for calf chymosin (Foltmann et al., 1978). Green (1972) observed that a crude extract from adult bovine stomach had a higher CU:PU than calf rennet. However, CU:PU for purified bovine chymosin is much lower than that for purified chymosin (Amer et al., 1980; deKoning, 1978). Gastricsin also has activities on milk and on hemoglobin, but there is little data on its CU:PU ratio. However, Tang (1970) reported that the milk-clotting and proteolytic activities of human gastricsin are less than those of porcine pepsin.

The optimum pH of hemoglobin hydrolysis for seal protease A was 3.5 (Figure 12) which is also similar to that for calf chymosin (Berridge, 1945). The pH optimum for protease C was 2.2, which is similar to those for human pepsin (Tang, 1970), porcine pepsin (Chiang et al., 1967), and bovine pepsin (Amer et al., 1980). An ion-exchange fraction of adult bovine gastric proteases with CU:PU similar to that of calf chymosin (adult bovine chymosin) had a pH optimum of 4 on hemoglobin substrate (Amer et al., 1980). A gastric protease from young rats was called chymosin based on its pH optimum (3.8–4.2) for hydrolysis of hemoglobin (Kotts and Jenness, 1976). Piglet chymosin (based on immunological activity and CU:PU) also had a pH optimum around 3.5 (Foltmann et al., 1978). Gastricsins hydrolyse hemoglobin optimally at pH 3.0 (Chiang et al., 1981; Ward et al., 1978; Chiang and Ponce, 1981; and Tang et al., 1967).

Stability and milk-clotting activity near neutral pH are considered distinguishing characteristics of chymosin (Malpress, 1967; Ernstrom, 1961; Foltmann, 1959). Although seal gastric protease A was more unstable than calf chymosin in buffers of various pH values, (Figure 13), it was much more stable at pH 7.0 than porcine pepsin. Seal proteases A and C exhibited milk-clotting activity up to pH 7.02 whereas under similar conditions porcine pepsin failed to clot milk above pH 6.5 (Figure 6). More recent reports show however, that other gastric proteases also have these properties. Fox (1969) showed that bovine pepsin clots milk up to pH 6.9. Chicken pepsin (Donta and Van Vunakis, 1970), porcine pepsin B (Ryle, 1970), gastricsins from fish (Chiang and Ponce, 1981), and toad (Ward et al., 1978) are stable in neutral or slightly alkaline buffers. Protease A and calf chymosin

were denatured by urea to the same extent in 90 minutes although the initial rates of inactivation were different. Protease C and porcine pepsin underwent much less inactivation. These observations are consistent with the view that hydrogen bonds may play more important role in maintaining the active configuration of seal protease A and of calf chymosin than of pepsin (Ernstrom, 1974).

Calcium chloride added to reconstituted skim milk influenced the clotting activities of both calf chymosin and protease A similarly (Figure 9). The optimum concentration of added calcium chloride was 0.02M for both the enzymes; above that concentration milk clotting was inhibited. This observation was consistent with the reported inhibition of the action of chymosin on κ-casein (Kanamori et al., 1977). However, Gordin and Rosenthal (1978) observed only slight inhibition of milk-clotting activities of chicken pepsin and calf rennet at high concentrations of added calcium chloride. The discrepancy between the results of this study and those reported by Gordin and Rosenthal may be due to differences in the conditions of milk-clotting assay as discussed earlier (Results and Discussion, Section VI(f)).

III. Conclusion: Tables 17, and 18 compare the properties of calf chymosin and protease A. Although there are some differences between the two enzymes most of the evidences support the hypothesis that protease A is chymosin-like. One notable difference is the lack of stability of protease A in buffers of various pH values particularly in pH values near 7 where chymosin is relatively stable (Foltmann, 1959). This was considered as a basis for distinction between calf chymosin and porcine pepsin at least in one instance to conclude that

Table 17. Comparison of the physical-chemical properties of protease A and other gastric proteases

Properties	Chymosin-like	Pepsin-like	Gastricsin-like
Molecular weight	similar[1]	different	similar
Amino acid composition	similar	different	different[2]
Isoelectric point	similar	different	--[3]
Electrophoretic mobility	partly similar	partly similar	--[3]
Stability in 6M urea	similar	different	--[3]
Stability in buffers of various pH values	different[4]	similar	similar[5]

[1] By SDS PAGE.

[2] Mills and Tang (1967).

[3] Data not available.

[4] More stable than porcine pepsin at pH 7.

[5] Fish gastricsin is an exception (Chiang and Ponce, 1981).

Table 18. Comparison of proteases A and other acidic proteases

Properties	Chymosin-like	Pepsin-like	Gastricsin-like
CU:PU	similar	different	--[1]
p^H optimum for Hb hydrolysis	similar	different	similar
Time-course of NPN release from casein	similar	different	--[1]
Inactivation of ribonuclease	similar	different	different
Activity on APDT	similar	different	similar
Sensitivity of milk-clotting activity to Ca^{++}	similar	different[2]	--[1]
Clotting activity as a function of milk p^H	similar	different	--[1]
Casein degradation in cheese	partly similar	different[3]	--[1]
Milk-clotting activity as a function of temperature	partly similar	partly similar	--[1]
Activation energy	partly similar	partly similar	--[1]
Milk-clotting activity as a function of enzyme dilution	different	similar	--[1]

Cord:

1. Data not available

2. Gordin and Rosenthal(1978) observed slight difference between calf chymosin and chicken pepsin with respect to their sensitivity to Ca^{++}.

3. SStanley and Emmons (1977) differences in the electrophoretic patterns of casein in Cheddar cheeses made with calf rennet and bovine pepsin.

human stomach does not secrete chymosin (Malpress, 1967). However, as discussed in the introduction, lack of stability in buffers of certain pH values does not rule out the possibility of existence of chymosin (Hirsch-Marie et al., 1976). The broad pH optimum of protease A for hemoglobin substrate is similar to both chymosins (pH optima 3.5-4.2) and gastricsins (pH optima 3.0). This, coupled with the very low hydrolytic activity of protease A on APDT and the low molecular weight of zymogen A estimated by HPLC and gel filtration raise the question whether protease A is a chymosin or gastricsin. However, unlike chymosin and seal gastric protease A, gastricsin has been classified as a minor gastric protease (Foltmann and Pedersen, 1977). Besides, gastricsins from pig and human inactivate ribonuclease (Tang, 1970) whereas seal protease A and chymosins from calf, lamb, kid and piglet do not (Bang-Jensen et al., 1964; O'Leary and Fox, 1975). The last named authors concluded that ribonuclease test is generally applicable to differentiate chymosin from other gastric proteases.

Based on the evidence discussed above, it may be concluded that seal gastric protease A is a chymosin-like enzyme. However, in view of the difficulty in defining an enzyme as chymosin as distinct from the homologous enzymes like pepsin and gastricsin, further studies, e.g., immunological relationship between protease A and other homologous gastric proteases (Foltmann, et al., 1981) and the comparison of its primary structure with those of the other gastric proteases (Foltmann and Pedersen, 1977) would confirm whether protease A is a chymosin or not.

IV. Cheese making properties of protease A and SGP: The properties of crude SGP and protease A with respect to their application as milk

coagulants for making cheese are comparable to those of calf rennet (Table 19). Like calf chymosin, the desirable component in commercial rennet, SGP clots milk near pH 7 and has limited specificity. A combination of these properties is not found in other rennet substitutes. Curdling of cheese milk at its physiological pH (near 7) without prior acidification is often practised by cheese industry, particularly to avoid phage contamination (Phelan, 1973). Most pepsins do not clot milk at pH 7; others can clot milk at neutral pH but are too proteolytic and are therefore undesirable for yield, texture and flavor considerations of the aged cheese.

In the actual cheesemaking trials the yield of cheese and fat retention were similar in cheeses made with calf rennet and SGP. The sensory preference scores of the cheeses made with crude SGP and protease A were either higher than or comparable to that prepared with calf rennet (Table 20). Protein degradation in the cheeses made with SGP and protease A was low as evident from the low amino acid contents and ripening indices of the citrate-HCl extracts of the cheeses made with these two coagulants. These observations suggest that limited proteolysis is desirable for development of sensory qualities of cheese, and are consistent with the literature reports that excessive proteolysis is associated with poor quality cheese.

In view of the similarity of calf chymosin and protease A in enzymatic and physicochemical properties and the results of the Cheddar cheese preparation with respect to yield, chemical and sensory analyses it may be concluded that seal gastric protease A and the crude SGP containing protease A may be a good rennet substitute. However, further trials, with larger number of cheese samples on an industrial

Table 19. Some criteria for an ideal rennet.

	Chymosin	SGP	Pepsin	Gastricsin
Ability to clot milk at p^H 7	+	+	-[1]	--[2]
Yield of cheese	+	+	-3	--[2]
Fat-retention	+	+	-3	--[2]
Specificity	+	+	-	--[2]
Availability	-	+[4]	+	-

1. Bovine and chicken pepsins are exceptions
2. Data not available
3. From reports on bovine(Emmons et. al., 1978) and chicken (Stanley et. al., 1980) pepsins.
4. Seal stomach should be easily available as a by-product of seal fishery in Newfoundland.

Table 20. Summary of the characteristics of the aged Cheddar cheeses

Coagulant	Sensory score	Free amino acids µmole/g	Total amino acids in citrate-HCl ext. µmole/g	Ripening index[1]	Degradation of α_{s1} casein[2]	Degradation of β casein[2]
Crude SGP	6.83	32.7	220.1	0.160	22.9	20.8
Protease A	5.83	33.0	209.4	0.167	23.1	21.0
Calf rennet	5.67	38.9	280.9	0.238	9.4	19.2
Fromase	5.90	35.1	315.3	0.267	18.8	12.6

[1]Expressed as the ratio of citrate-HCl soluble protein to total protein in cheese (determined by Kjeldahl method).

[2]Residual casein as a percentage of total protein after 30 weeks of aging.

scale will be necessary to conclusively establish the suitability of seal gastric proteases as rennet substitutes.

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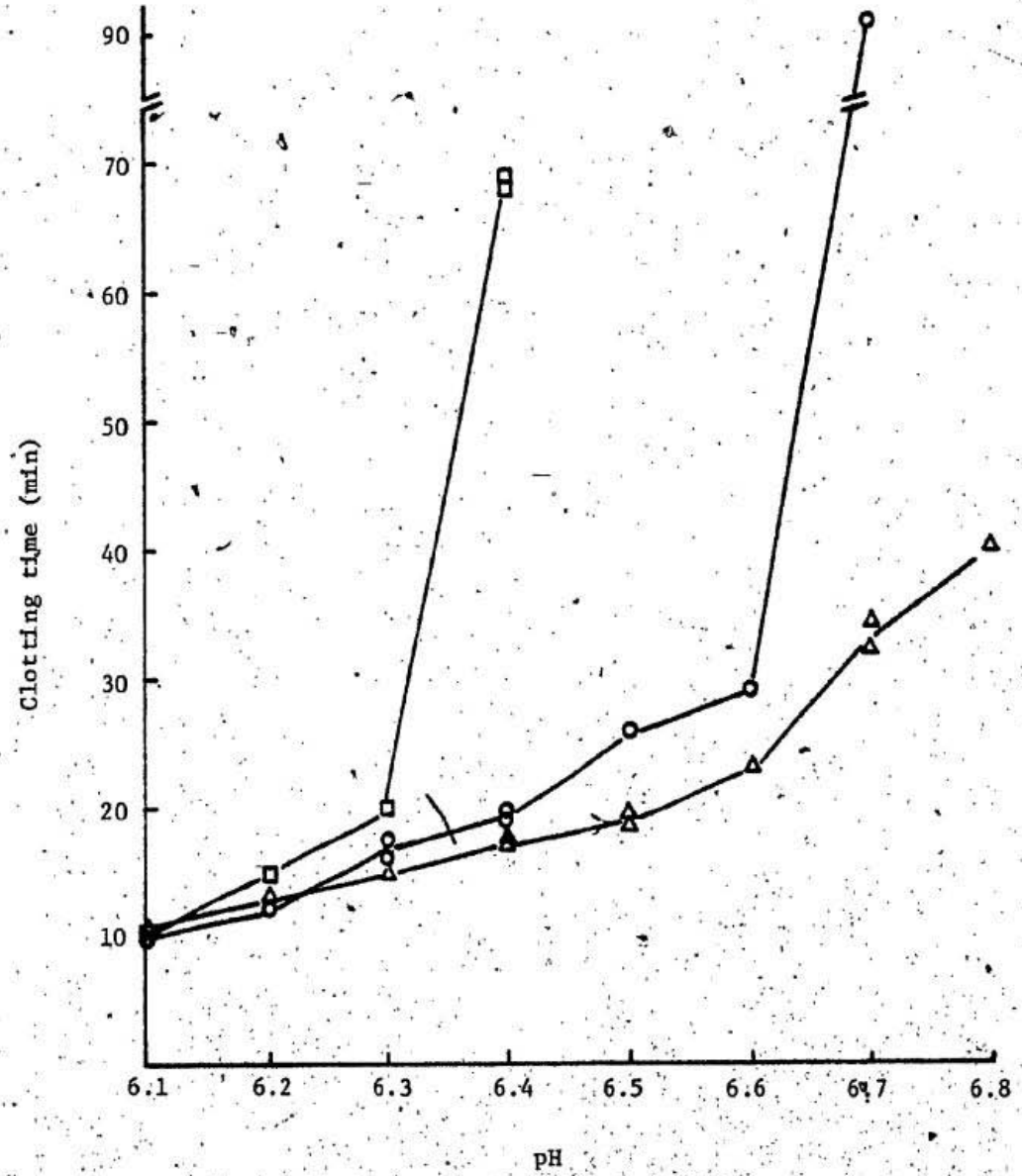
Appendix A. Influence of pH on milk-clotting times of the enzymes

Enzyme concentrations were adjusted to have equal milk clotting times at pH 6.1. The milk pH was adjusted with 1 M NaOH or HCl. Porcine pepsin, □ ; seal gastric protease, ○; and calf chymosin, △.


Clotting time (min)
90
70
60
50
40
30
20
10
6.1
6.2
6.3
6.4
6.5
6.6
6.7
6.8
pH

Appendix B. Relative rates of milk-clotting and proteolytic activities of proteases.

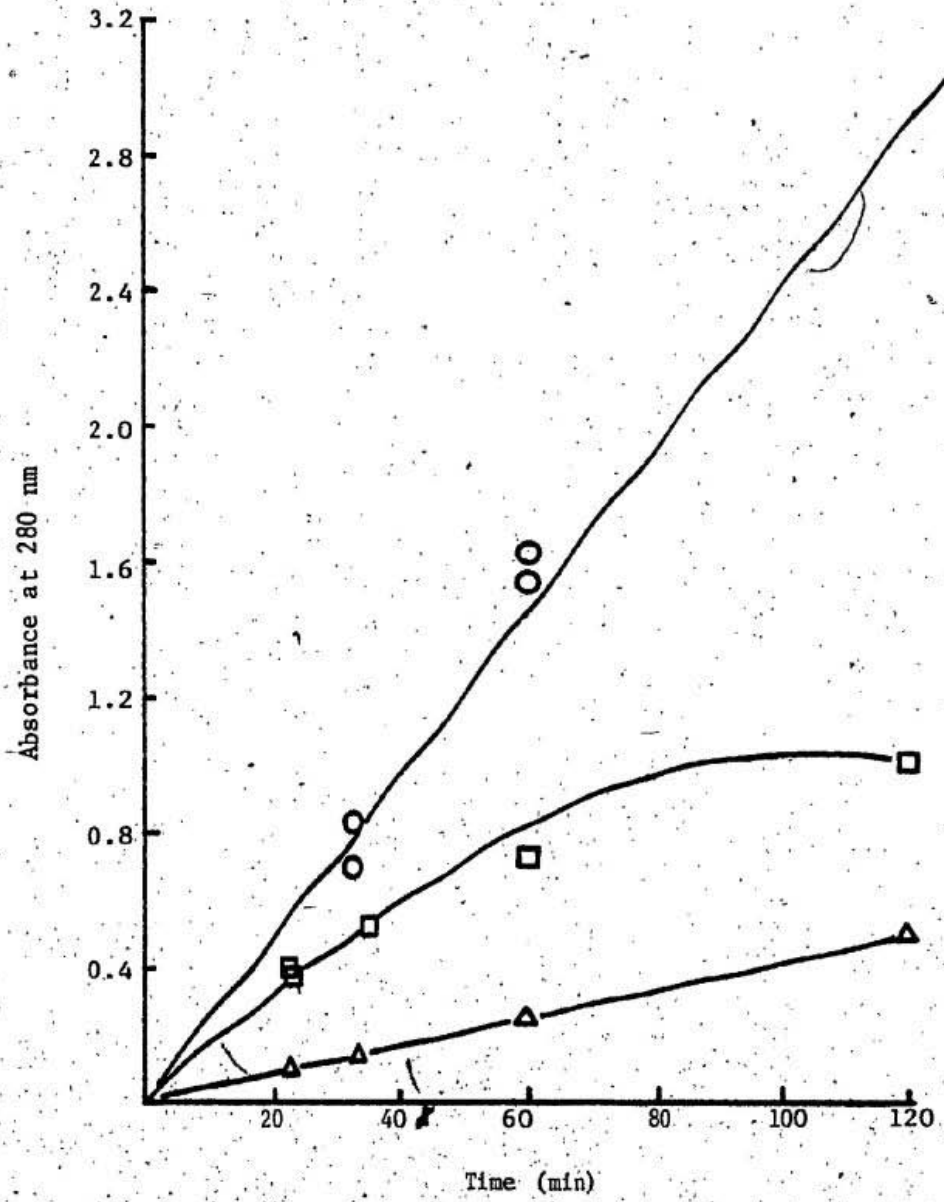
Protease	Substrate	
	Hemoglobin CU:PU[1]	Casein CU:PU
Calf chymosin	0.170	1.61
Porcine pepsin	0.005	0.39
SGP	0.025	0.26

1 Pepsin unit (PU) and clotting unit (CU) are as defined by Anson (1948) and Berridge (1945). The pepsin units are based on initial velocity.

Appendix C. Time-course of casein hydrolysis by proteases.

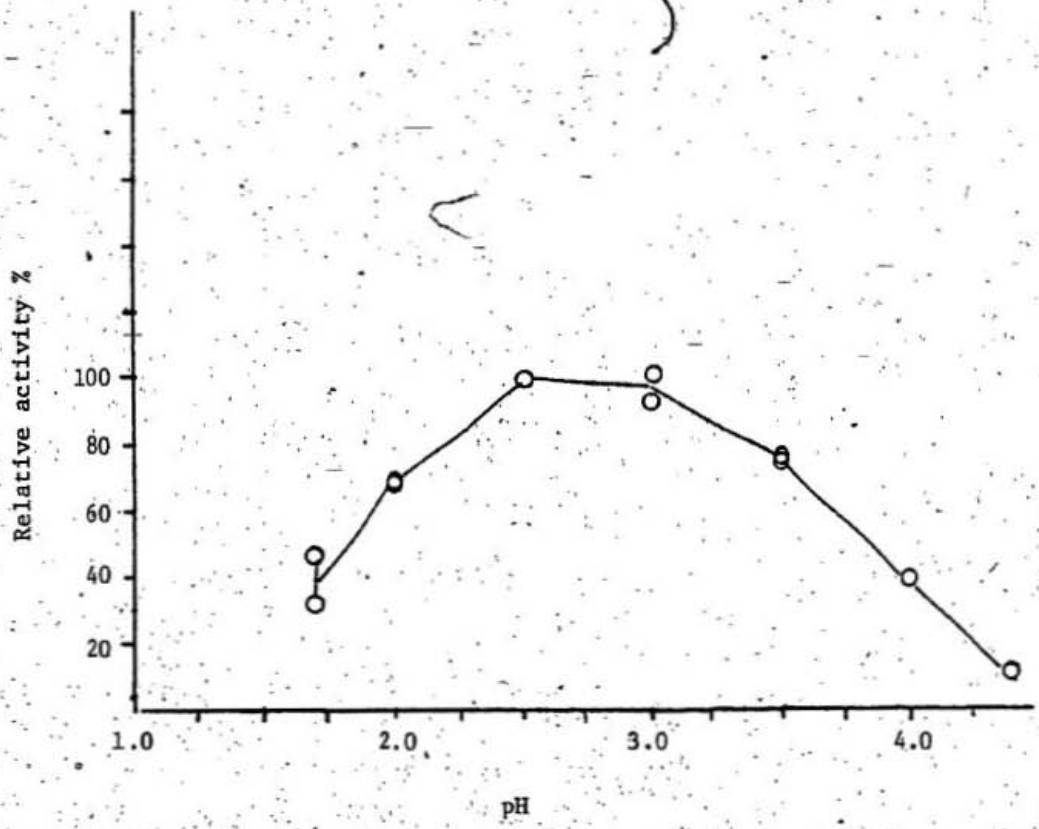
Ten ml of a 2% casein solution was incubated with 0.5 ml of each of the three proteases (porcine pepsin, crude seal gastric protease, and calf chymosin) at 37°C. At intervals 1.0 ml aliquots were withdrawn in duplicate, treated with 1.0 ml of 10% TCA. The mixture was cooled at 4°C for 30 minutes and centrifuged at 3,000xg for 30 minutes. The absorbance at 280nm was measured as described in Materials and Methods.

Porcine pepsin, □ ; SGP, ○ ; calf chymosin, △ .


3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
Absorbance at 280 nm
20
40
60
80
100
120
Time (min)

Appendix D. Influence of pH on the hydrolysis of hemoglobin by crude SGP.

Three ml of 2% hemoglobin in citrate buffers of various pH values were equilibrated at 30°C. To each sample 0.2 ml of crude SGP containing 3.9 pepsin units of activity was added. After 20 minutes 0.9 ml samples were withdrawn, mixed with 1.0 ml of 6% TCA, cooled on ice for 30 minutes and centrifuged at 3,000 x g for 30 minutes. The A280 nm of the supernatant was noted. The A280 nm of the control samples in which the enzyme was added after the TCA was subtracted from the corresponding test samples.


Relative activity %
100
80
60
40
20
1.0
2.0
3.0
4.0
pH

Appendix E. Analysis of experimental Cheddar cheeses

Coagulant	Yield (g/100 ml milk)				
	Fresh Weight	Dry Weight	% moisture	Citrate-HCl Soluble N[1] $A_{274.5nm}$	Sensory quality[2]
SGP	9.35	6.60	29.3	1.73	Typical Cheddar
Calf rennet	8.64	6.25	27.6	1.48	Typical Cheddar

1 Average of duplicate measurements following the method of Vakaleris and Price (1959).

2 Based on tasting by 5 individuals in the laboratory after 11 weeks of aging. The cheeses showed mold growth when 17 weeks old and therefore were not suitable for a routine taste panel.

Appendix F. Free amino acid composition of citrate-HCl extracts from one-week old Cheddar cheeses

Amino Acid	Calf Rennet		Crude SGP		Protease A		Fromase	
	μmole/g	%	μmole/g	%	μmole/g	%	μmole/g	%
Cysteic acid	0.60	13.6	0.79	15.3	0.73	12.8	0.72	15.6
Glycerophospho-ethanolamine	0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.0
Aspartic acid	0.40	9.0	0.33	6.4	0.15	2.6	0.28	6.1
Threonine	0.05	1.1	0.04	0.8	0.09	1.6	0.07	1.5
Serine	0.04	0.9	0.05	1.0	0.07	1.2	0.07	1.5
Asparagine	0.07	1.6	0.24	4.6	0.19	3.3	Trace	---
Glutamic Acid	0.69	15.6	0.73	14.1	0.92	16.1	0.65	14.1
Glutamine	0.00	0.0	0.14	2.7	0.17	3.0	0.00	0.0
Proline	0.18	4.1	0.44	8.5	0.33	5.8	0.29	6.3
Glycine	0.09	2.0	0.07	1.4	0.07	1.2	0.07	1.5
Alanine	0.15	3.4	0.23	4.4	0.44	7.7	0.13	2.8
Citruline	0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.0
Valine	0.09	2.0	0.10	1.9	0.14	2.5	0.07	1.5
Cysteine	0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.0
Methionine	0.00	0.0	0.03	0.6	0.07	1.2	0.00	0.0
Cystathionine	Trace	---	0.00	0.0	0.00	0.0	0.00	0.0
Isoleucine	0.07	1.6	0.12	2.3	0.14	2.5	0.08	1.7
Leucine	0.74	16.7	0.51	9.8	0.41	7.2	0.67	14.5
Tyrosine	0.18	4.1	0.29	5.6	0.37	6.5	0.40	8.7
Phenylalanine	0.39	8.8	0.26	5.0	0.14	2.5	0.60	13.0
γ-Aminobutyric acid	0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.0
Tryptophan	0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.0
Ornithine	0.07	1.6	0.05	1.0	0.09	1.6	0.06	1.3
Lysine	0.31	7.0	0.49	9.5	0.88	15.4	0.23	5.0
Histidine	0.07	1.6	0.10	1.9	0.07	1.2	0.07	1.5
Arginine	0.23	5.2	0.17	3.3	0.24	4.2	0.15	3.3
Taurine	0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.0
β-alanine	0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.0
Total	4.42		5.18		5.71		4.61	

Appendix G. Free amino acid composition of citrate-HCl extracts from 30-week old Cheddar cheeses.

Amino Acid	Calf Rennet		Crude SGP		Protease A		Fromase	
	μmole/g	%	μmole/g	%	μmole/g	%	μmole/g	%
Cysteic acid	0.9	2.3	1.4	4.3	1.4	4.2	0.8	2.3
Glycerophospho-ethanolamine	1.5	3.9	1.1	3.4	1.5	4.5	1.3	3.7
Aspartic acid	1.6	4.1	1.5	4.6	1.4	4.2	0.9	2.6
Threonine	0.6	1.5	0.9	2.8	0.6	1.8	0.8	2.3
Serine	1.2	3.1	0.5	1.5	1.3	3.9	1.7	4.8
Asparagine	2.5	6.4	1.9	5.8	1.9	5.8	2.4	6.8
Glutamic Acid	4.4	11.3	4.2	12.8	4.6	13.9	4.3	12.3
Glutamine	1.4	3.6	2.2	6.7	1.8	5.5	1.8	5.1
Proline	0.7	1.8	0.4	1.2	0.7	2.1	0.8	2.3
Glycine	0.9	2.3	0.8	2.4	0.7	2.1	0.6	1.7
Alanine	1.0	2.6	1.5	4.6	1.4	4.2	1.0	2.8
Citruline	0.2	0.5	Trace	---	0.1	0.3	0.1	0.3
Valine	2.6	6.7	1.3	4.0	1.2	3.6	2.8	8.0
Cysteine	Trace	---	Trace	---	Trace	---	Trace	---
Methionine	1.0	2.6	1.2	3.7	1.2	3.6	1.4	3.9
Cystathionine	Trace	---	0.0	---	Trace	---	0.0	---
Isoleucine	0.5	1.3	0.4	1.2	0.5	1.5	0.6	1.7
Leucine	7.7	19.8	6.1	18.7	5.5	16.7	4.2	12.0
Tyrosine	1.4	3.6	1.4	4.3	1.3	3.9	1.5	4.3
Phenylalanine	4.3	11.1	2.3	7.0	2.2	6.7	3.8	10.8
γ-Aminobutyric acid	0.1	0.2	0.1	0.3	0.1	0.3	0.0	0.0
Tryptophan	0.1	0.2	0.1	0.3	0.1	0.3	0.1	0.3
Ornithine	0.3	0.8	0.1	0.3	0.2	0.6	0.4	1.1
Lysine	1.2	3.1	1.2	3.6	1.4	4.2	0.9	2.6
Histidine	0.2	0.5	0.2	0.6	0.2	0.6	0.2	0.6
Arginine	2.6	6.7	1.9	5.8	1.7	5.2	1.2	3.4
Taurine	---	---	0.0	---	0.0	---	0.0	0.0
β-alanine	---	---	---	---	---	---	1.5	4.3
Total	38.9		32.7		33.0		35.1	

Appendix H. Total amino acid composition of citrateHCl extracts from one-week old Cheddar cheeses[1].

Amino Acid	Calf Rennet		Crude SGP		Protease A		Fromase	
	μmole/g	%	μmole/g	%	μmole/g	%	μmole/g	%
Cysteic acid	Present	---	Present	---	Present	---	Present	---
Glycerophospho-ethanolamine	---	---	---	---	---	---	---	---
Aspartic acid	9.77	7.38	9.55	7.3	8.99	7.4	12.44	7.1
Threonine	4.16	3.1	4.08	3.1	3.56	3.0	5.58	3.2
Serine	8.96	6.7	7.76	5.9	7.40	6.1	9.91	5.7
Asparagine	---	---	---	---	---	---	---	---
Glutamic Acid	29.17	21.8	28.73	22.0	26.98	22.3	38.68	22.1
Glutamine	---	---	---	---	---	---	---	---
Proline	13.77	10.3	13.38	10.2	12.18	10.1	20.49	11.7
Glycine	4.10	3.1	4.02	3.1	3.74	3.1	4.76	2.7
Alanine	4.23	3.2	3.90	3.0	3.61	3.0	5.05	2.9
Citruline	---	---	---	---	---	---	---	---
Valine	9.37	7.0	9.44	7.2	8.78	7.3	13.72	7.8
Cysteine	0.13	0.0	0.00	0.0	0.00	0.0	0.00	0.0
Methionine	2.58	1.9	2.30	1.8	1.70	1.4	3.36	1.9
Cystathionine	---	---	---	---	---	---	---	---
Isoleucine	6.88	5.1	7.37	5.6	6.71	5.6	10.26	5.9
Leucine	12.56	9.4	13.07	10.0	11.82	9.8	17.17	9.8
Tyrosine	3.15	2.4	2.95	2.3	2.71	2.2	3.86	2.2
Phenylalanine	5.17	3.9	5.20	4.0	4.54	3.8	7.42	4.2
γ-Aminobutyric acid	---	---	---	---	---	---	---	---
Tryptophan	---	---	---	---	---	---	---	---
Ornithine	---	---	---	---	---	---	---	---
Lysine	11.44	8.6	10.76	8.2	10.49	8.7	12.36	7.1
Histidine	3.71	2.8	3.76	2.9	3.36	2.8	4.92	2.8
Arginine	4.53	3.4	4.57	3.5	4.16	3.4	5.28	3.0
Taurine	---	---	---	---	---	---	---	---
β-alanine	0.00	---	0.00	---	0.00	---	0.00	---
Total	133.68		130.84		127.70		175.26	

Note (a) Total amino acid (acid hydrolysate).

Appendix I. Total amino acid composition of the citrate-HCl extracts from 30-week old Cheddar cheeses.[1]

Amino Acid	Calf Rennet		Crude SGP		Protease A		Fromase	
	μmole/g	%	μmole/g	%	μmole/g	%	μmole/g	%
Cysteic acid	0.5	0.1	0.3	0.1	0.3	0.1	0.3	0.1
Glycerophospho-ethanolamine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Aspartic acid	22.3	7.9	16.1	7.3	16.0	7.6	23.2	7.4
Threonine	9.8	3.5	6.8	3.1	6.6	3.1	12.0	3.8
Serine	17.9	6.4	13.2	6.0	13.0	6.2	20.4	6.5
Asparagine	*	---	*	---	*	---	*	---
Glutamic Acid	56.8	20.2	44.3	20.1	43.6	20.8	61.2	19.4
Glutamine	*	---	*	---	*	---	*	---
Proline	30.4	10.8	23.0	10.4	21.8	10.4	38.3	12.1
Glycine	8.9	3.2	6.5	3.0	6.5	3.1	8.6	2.7
Alanine	9.1	3.2	6.2	2.8	6.2	3.0	8.5	2.7
Citrulline	---	---	---	---	---	---	---	---
Valine	13.0	4.6	22.1	10.0	14.7	7.0	23.1	7.3
Cysteine	0.3	0.0	0.2	0.0	0.2	0.0	0.4	0.1
Methionine	4.8	1.7	3.6	1.6	3.4	1.6	6.1	1.9
Cystathionine	0.1	0.0	0.0	0.0	0.0	0.0	0.1	---
Isoleucine	16.0	5.7	11.3	5.1	11.6	5.5	17.5	5.6
Leucine	26.4	9.4	20.5	9.3	20.1	9.6	28.4	9.0
Tyrosine	9.2	3.3	6.5	3.0	6.7	3.2	9.9	3.1
Phenylalanine	11.3	4.0	8.2	3.7	7.7	3.7	13.1	4.2
γ-Aminobutyric acid	---	---	---	---	---	---	---	---
Tryptophan	---	---	---	---	---	---	---	---
Ornithine	0.2	0.0	0.1	0.0	0.1	0.0	0.3	0.0
Lysine	24.2	8.6	16.6	7.5	17.4	8.3	24.6	7.8
Histidine	7.9	2.8	5.8	2.6	5.6	2.7	8.4	2.7
Arginine	9.5	3.4	7.1	3.2	5.6	2.7	9.1	2.9
Taurine	2.3	0.8	1.7	0.8	2.3	1.1	1.8	0.6
β-alanine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Total	280.9		220.1		209.4		315.3	

Note: 1 Total amino acids (Hydrolysate).
*Converted to corresponding acids during HCl hydrolysis.

Appendix J. Amino acid composition of citrate-HCl extracts from "Forfar" Cheddar cheese.

Amino acid	Free μmole/g	%	Total μmole/g	%
Cysteic acid	7.7	3.8	0.9	0.1
Glycerophospho-ethanolamine	0.0	0.0	0.0	0.0
Aspartic acid	3.3	1.6	17.3	2.8
Threonine	7.3	3.6	22.8	3.7
Serine	4.9	2.4	33.4	5.4
Asparagine	11.6	5.7	*	-
Glutamic Acid	36.7	17.9	144.4	23.3
Glutamine	7.1	3.5	*	-
Proline	5.2	2.5	68.1	11.0
Glycine	6.3	3.1	19.5	3.2
Alanine	7.7	3.8	20.0	3.2
Citrulline	3.2	1.6	1.5	0.2
Valine	13.4	6.5	46.4	7.5
Cysteine	0.2	0.0	2.1	0.3
Methionine	6.1	3.0	10.7	1.7
Cystathionine	0.0	0.0	0.3	0.0
Isoleucine	5.0	2.4	36.1	5.8
Leucine	33.5	16.3	52.9	8.5
Tyrosine	7.6	3.7	15.1	2.4
Phenylalanine	11.0	5.4	24.9	4.0
γ-Aminobutyric acid	0.4	0.2	0.1	0.0
Tryptophan	0.7	0.3	0.0	0.0
Ornithine	4.4	2.1	5.6	0.9
Lysine	16.2	7.9	53.9	8.7
Histidine	2.6	1.3	18.4	3.0
Arginine	2.9	1.4	13.4	2.2
Taurine	0.0	0.0	11.0	1.8
β-alanine	0.0	0.0	0.0	0.0
Total	205.0		618.8	

*converted to corresponding acid during HCl hydrolysis.

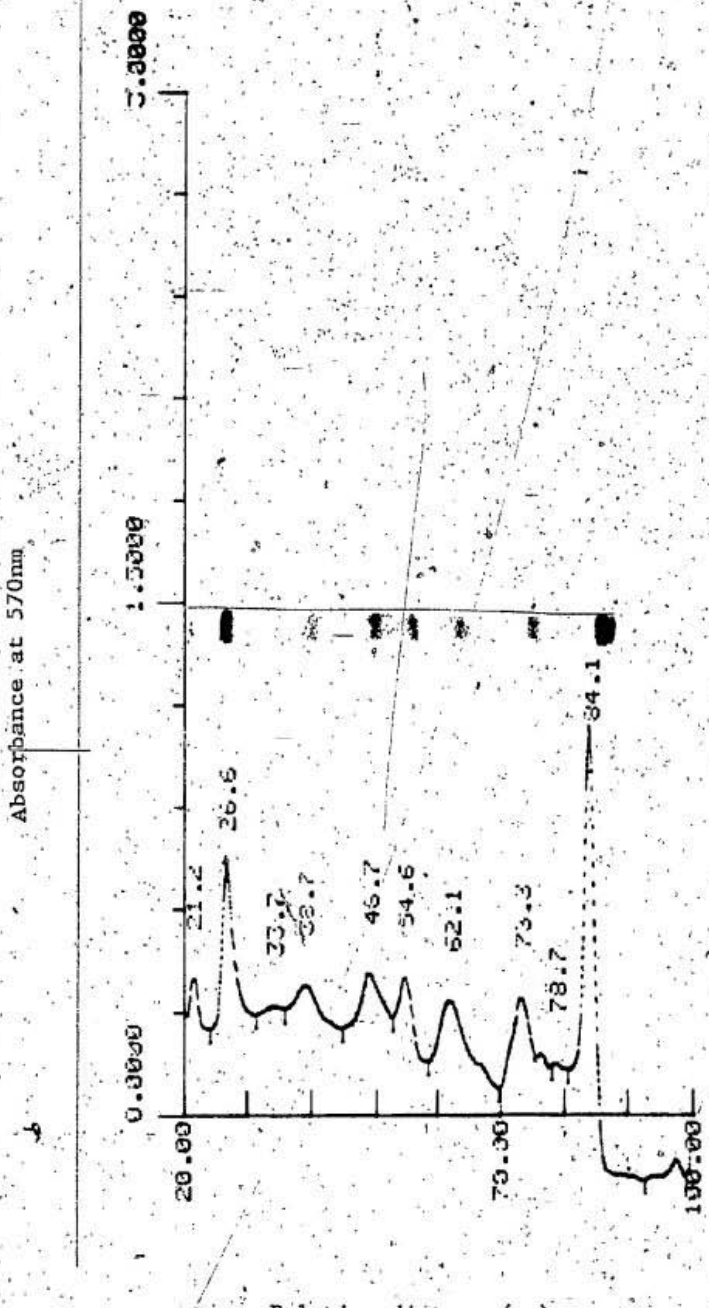
Appendix K. Amino Acid Composition of Whole Casein

Amino Acid	Percentage Composition
Aspartic Acid	8.8
Methionine sulphone	0.0
Threonine	4.6
Serine	6.9
Glutamic Acid	18.3
Proline	9.8
Glycine	3.0
Alanine	4.3
Valine	6.1
Cysteine	0.5
Methionine	4.1
Cystathionine	0.0
Isoleucine	4.5
Leucine	9.2
Tyrosine	4.3
Phenylalanine	3.8
Ornithine	0.0
Lysine	6.8
Histidine	2.3
Arginine	2.6

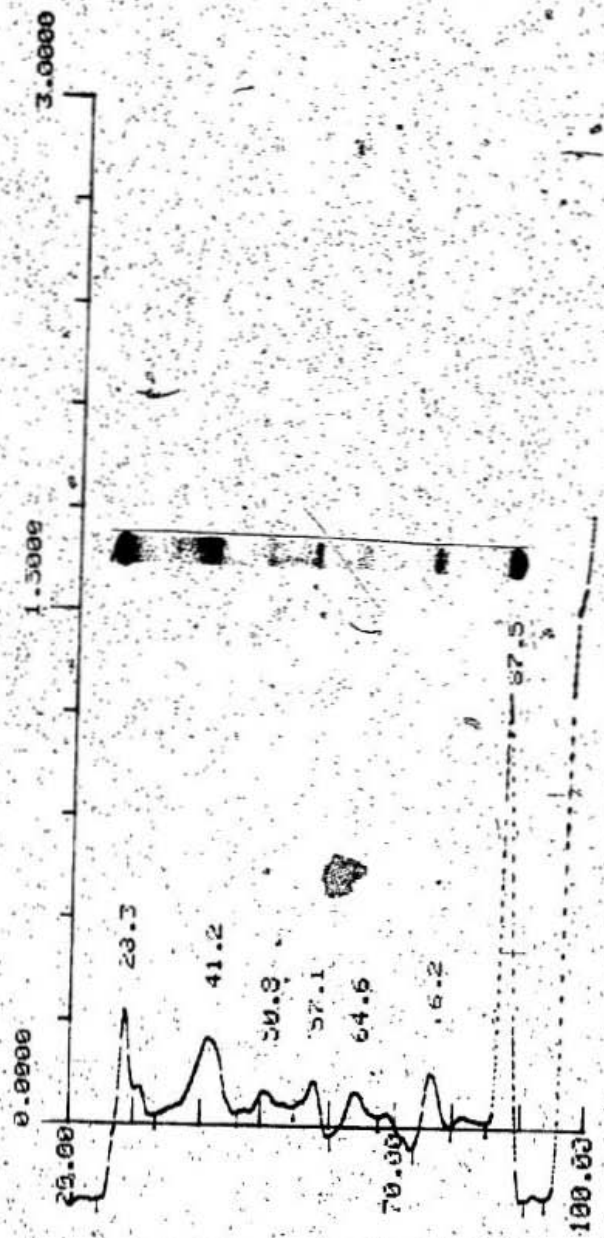
Appendix L. Protein band patterns of citrate-HCl extracts.

Citrate-HCl extracts of 30-week-old cheeses were prepared following the method of Vakaleris and Price (1959) and 10 µl of extracts were subjected to polyacrylamide gel electrophoresis at pH 8.3 (Davis, 1964) and the gels stained with coomassie blue and destained. The gels were then scanned in DU8 spectrophotometer. (a) extract from cheese made with protease A, (b) crude SGP, (c) calf rennet, (d) Fromase.

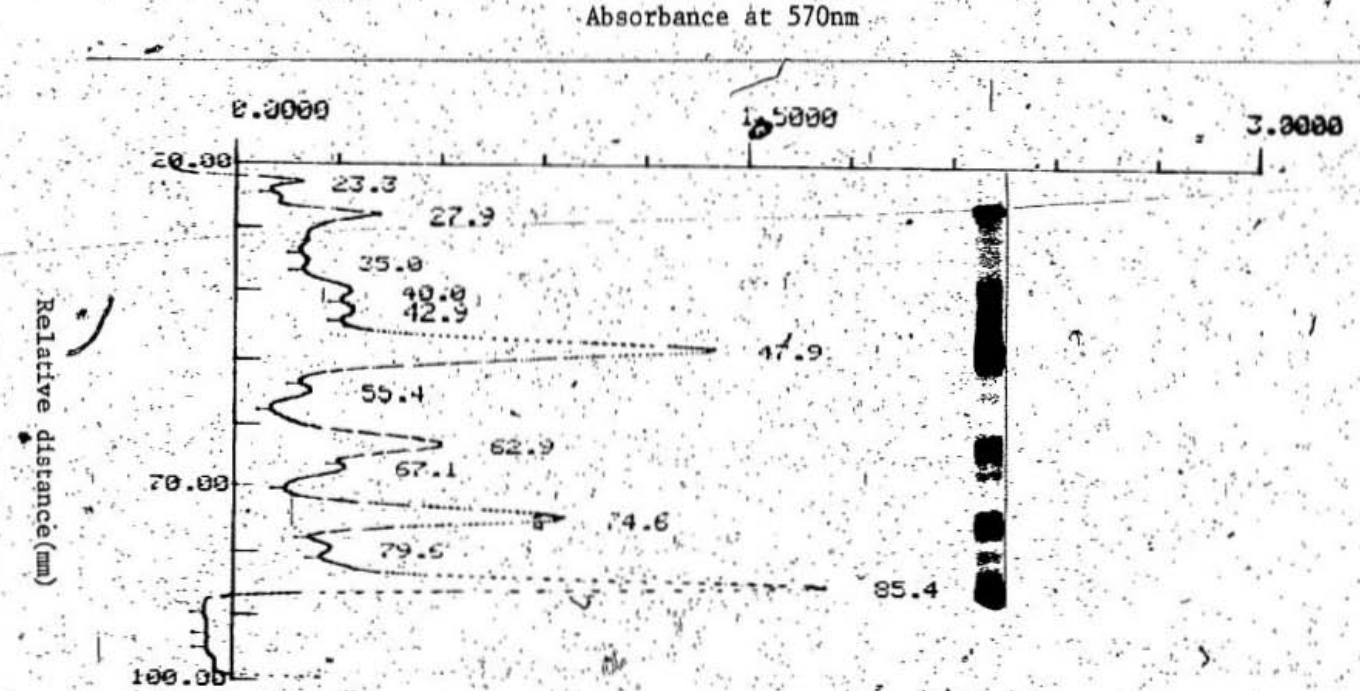
(a)


Absorbance at 570nm
1.5000
0.0000
20.00
75.00
100.00
21.2
26.6
33.7
38.7
46.7
54.6
62.1
73.3
78.7
84.1
Relative distance(mm)

(b)



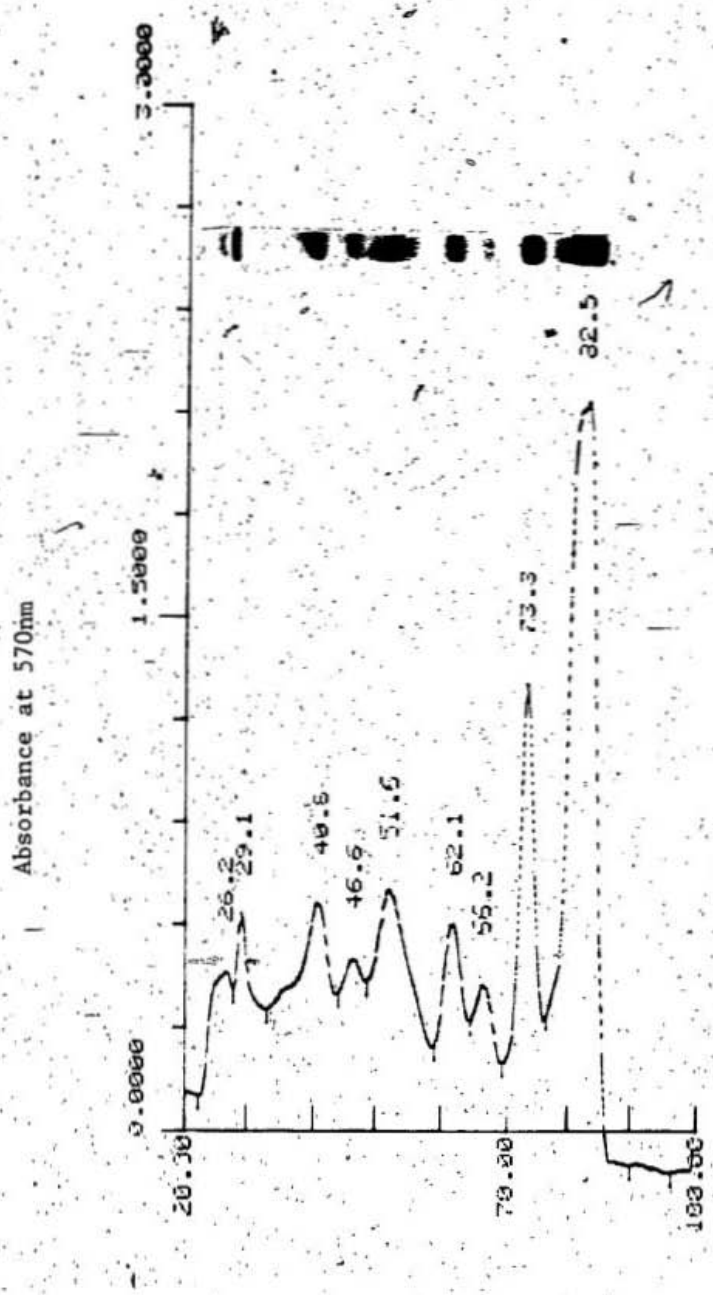
Relative distance(mm)

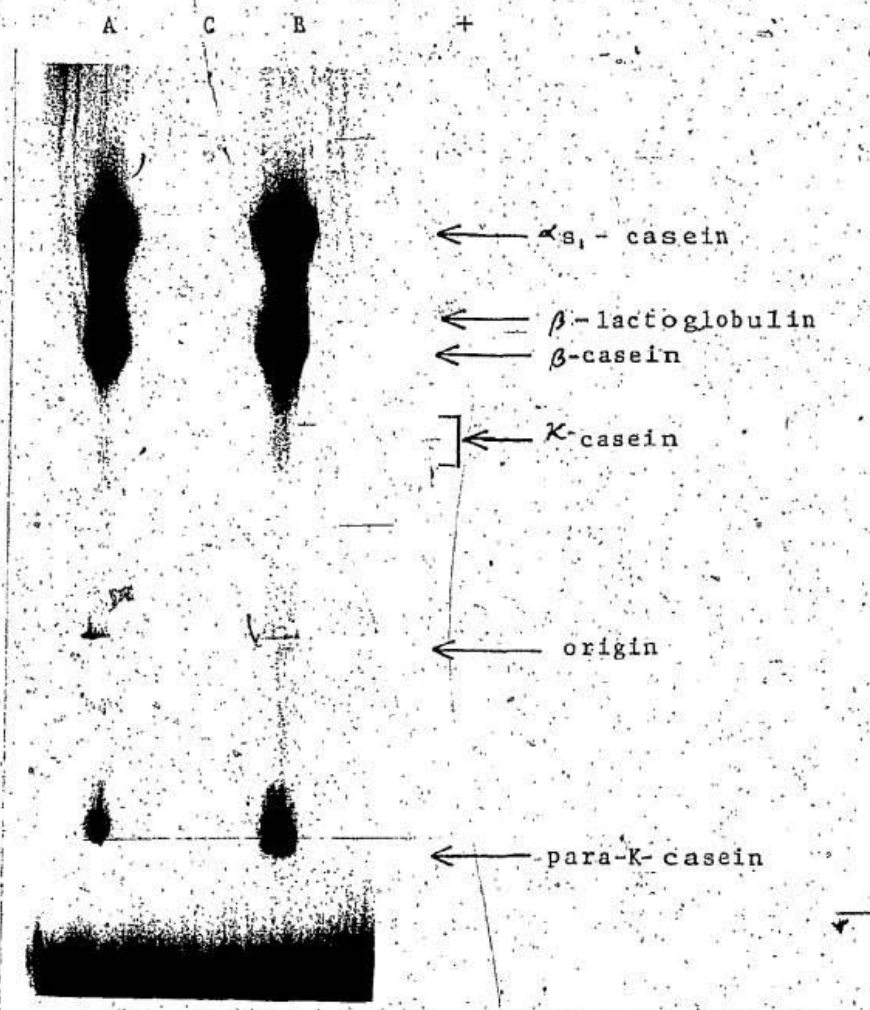

Absorbance at 570nm
0.0000
1.5000
3.0000
20.00
23.3
27.9
35.0
40.0
42.9
47.9
55.4
62.9
67.1
70.00
74.6
79.5
85.4
100.00
Relative distance(mm)

185

(e)

(d)


3.0000
1.5000
0.0000
Absorbance at 570nm
26.2
29.1
40.6
46.6
51.5
62.1
66.2
73.3
82.5
20.30
79.20
103.50
Relative distance(mm)



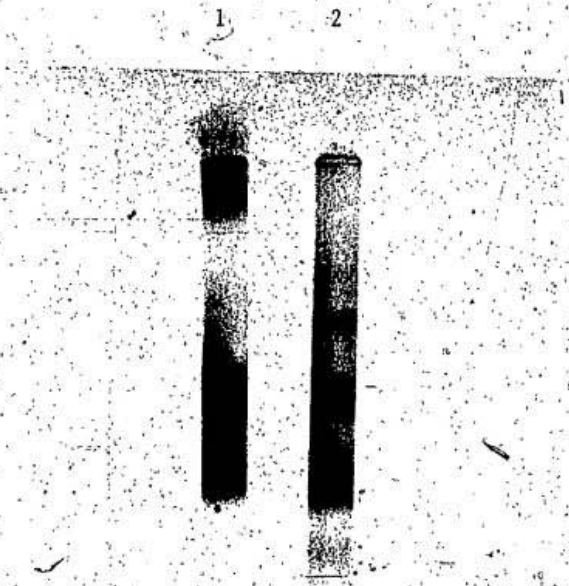
Appendix M. Starch gel electrophoresis of Berridge substrate clotted by calf chymosin and protease A.

A 0.5 ml sample of Berridge substrate, equilibrated at 30°C was treated with calf chymosin or protease A so that the substrate would clot in about 10 minutes. The clotted substrate was then dissolved in urea and analysed by starch gel electrophoresis according to Green (1972). A, calf chymosin-treated, and B, protease A-treated Berridge substrate, C, Protease A-control.

Appendix N. Polyacrylamide gel electrophoresis of zymogen A and its active form.

Polyacrylamide gel electrophoresis in the absence of sodium dodecyl sulphate was performed as described in Materials and Methods.

1, zymogen A purified by affinity chromatography; 2, protease A prepared by activation of zymogen A.


1
2

Appendix Q. Sensory preference scores of the Cheddar Cheeses after 4 weeks of aging*

Judge Number	Protease A.	Control rennet	Fromase	Crude SGP
1	8	7	7	8
2	6	7	7	4
3	5	5	5	5
4	7	6	5	4
5	7	6	6	5
6	7	7	8	7
7	7	6	6	8
8	6	8	5	8
9	8	4	4	9
10	7	6	8	7
11	8	3	3	7
12	6	8	8	9
13	6	8	5	4
14	8	6	3	4
15	7	6	4	6
16	8	2	2	5
17	8	7	5	8
18	5	8	7	6
19	7	3	4	6
20	7	8	9	6
21	5	6	4	5
22	6	8	6	7
23	7	7	5	5
24	8	7	5	7
25	4	5	5	4
26	8	9	7	7
27	7	6	4	3
28	7	6	8	4
29	8	7	6	3
30	8	9	4	2
Mean ± S.D.	6.87±1.11	6.37±1.71	5.50±1.73	5.77±1.87
Liked by	26	24	13	16
Neither liked nor disliked	3	2	8	5
Disliked by	1	4	9	9

*Rated on a 9 point hedonic scale.

Appendix P. Sensory preference scores* of 30-week-old Cheddar cheeses

JUDGE NUMBER	Protease A	Control rennet	Fromase	crude SGP
	sensory score			
1	5	5	6	6
2	7	6	7	9
3	8	8	8	8
4	8	9	8	6
5	3	7	2	7
6	4	8	6	3
7	7	3	6	8
8	2	7	6	9
9	6	4	6	8
10	6	6	5	7
11	5	3	4	9
12	5	2	5	7
13	4	8	6	7
14	5	2	6	8
15	6	6	4	7
16	8	5	5	5
17	7	4	8	9
18	3	3	2	7
19	8	6	7	5
20	5	6	7	4
21	6	7	7	8
22	4	7	6	3
23	7	8	7	8
24	6	5	5	7
25	8	4	7	7
26	7	7	7	6
27	6	7	5	8
28	7	7	7	7
29	5	3	5	5
30	7	7	7	7
Mean ± S.D.	5.83±1.64	5.67±1.97	5.90±1.52	6.83±1.64
Liked by	18 Judges	18 Judges	20 Judges	24 Judges
Neither liked/ Disliked by	6 Judges	3 Judges	6 Judges	3 Judges
Disliked by	6 Judges	9 Judges	4 Judges	3 Judges

*Rated on a 9 point hedonic scale.

Appendix .Q. Questionnaire for hedonic scale

Name _____ Date _____

You are provided with samples of Cheddar cheeses. Taste the samples and check how much you like or dislike each one.

__like extremely	__like extremely	__like extremely	__like extremely
__like very much	__like very much	__like very much	__like very much
__like moderately	__like moderately	__like moderately	__like moderately
__like slightly	__like slightly	__like slightly	__like slightly
__neither like or dislike	__neither like or dislike	__neither like or dislike	__neither like or dislike
__dislike slightly	__dislike slightly	__dislike slightly	__dislike slightly
__dislike moderately	__dislike moderately	__dislike moderately	__dislike moderately
__dislike very much	__dislike very much	__dislike very much	__dislike very much
__dislike extremely	__dislike extremely	__dislike extremely	__dislike extremely

Comments: