CHEMICAL MODIFICATION OF CARBOXYL GROUPS IN
PORCINE PEPSIN

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ABSTRACT

Carboxyl groups in porcine pepsin were chemically modified by the carbodiimide reaction using water-soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and amino acid esters as nucleophiles. The modification resulted in profound changes in the activities, specificity and some physico-chemical properties of the enzyme. These include: (1) significant decrease in milk clotting activity without changes in proteolytic activity against hemoglobin; (2) decrease in peptidase activity against N-acetyl-L-phenylalanyl-diiodo-L-tyrosine; (3) increase in clotting activity against κ -casein but decrease in clotting activity against κ - α_{s1} -casein mixture; (4) shift in proteolytic pH profile with pH optimum increased from 2.0 to about 3.5; (5) decrease in relative electrophoretic mobility and a slight decrease in isoelectric point; (6) increase in K_m without much change in k_{cat} ; and (7) increase in stability at pH above 6.0.

Results suggest that the drop in milk clotting activity was due to a change in the charge distribution on the enzyme affecting enzyme-micelle interaction.

The presence of dipeptide substrates interfered with the carboxyl modification suggestive of the proximity of the modified groups to the enzyme active site.

The modified enzyme remained reactive to site-specific inactivators but at rates slower than the native enzyme. The modification was not specific, causing similar changes in pepsinogen and chymosin.

The modified and native pepsins had similar caseinolytic properties and produced comparable rates of syneresis and curd tension development on curdled milk. The increase in pH stability suggested that the modified enzyme may be a better calf rennet substitute than native pepsin for cheesemaking.

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INTRODUCTION

Chemical modification of proteins by specific reagents is a useful technique in the study of the physico-chemical basis and mechanisms of protein function in the biological systems (Means and Feeney, 1971). In the food industry, chemical modification is playing an increasingly important role. Many food proteins, particularly those from non-conventional sources, have been modified to improve their functional properties. Alkali treatment and modification with various acylating agents are the two most widely used methods to alter the functionality of food proteins (Kinsella, 1976; Ryan, 1977). A large number of patents have appeared, and in most published cases, improvements in functional properties were obtained making it possible to extend and replace existing food proteins with novel proteins in processed food and for the fabrication of new foods.

Chemical modification of enzymes is usually undertaken to elucidate the action mechanism and active-site residues of the biological catalysts. Immobilization of enzymes, which is one form of chemical modification, has been extensively utilized in bio-medical research as well as in the food industry. The feasibility of modifying food-related enzymes to conform to particular processing requirements or to avoid

undesirable characteristics has not been explored, although it should hold great promises in enhancing the applicability of many enzymes in food manufacture.

The aim of the present investigation is to determine whether the performance of a food-related enzyme can be improved by chemical modification. Porcine pepsin (E.C. 3,4,23,1), an acid protease found in the stomach mucosa of pig, is chosen for the following reasons: (1) Porcine pepsin is the most widely accepted milk coagulant from an animal source as a substitute for calf rennet in cheese-making, and has been used as 50:50 mixtures with calf rennet in the cheese industry (Phelan, 1973; Bottazzi et al., 1976; Carbone and Emaldi, 1976). (2) The use of swine pepsin alone is generally considered unsuitable for cheese-making (Sardinas, 1972). This has been partly attributed to slower proteolysis in cheeses made with swine pepsin than in those made with calf rennet (Green and Foster, 1974). Porcine pepsin is known to be less stable than chymosin (major enzyme in calf rennet) at pH above 6.0 (Herriott, 1955; Foltmann, 1966; Antonini and Ribadeau Dumas, 1971), suggesting that it may be inactivated to a greater extent than rennet during the cheese-making process. (3) Porcine pepsin has been the most thoroughly studied among all acid proteases. The primary structure was determined (Tang et al., 1973), and its properties and modes of action were well characterized.

The primary objective of the project is to modify selectively the reactive groups of the amino acid residues in pepsin with specific chemical reagents so as to change the activity, stability and physicochemical properties of the enzyme. These alterations, if beneficial, should enhance the potential utilization of porcine pepsin in the manufacture of cheese. Preliminary experiments had been carried out to modify arginine, tryptophan, tyrosine and carboxyl groups in pepsin. Carboxyl modification was found to be most promising and was further investigated.

The enzymatic coagulation of milk is a complex process involving a primary proteolytic phase and a secondary aggregation phase. The mechanism by which micelles aggregate to form curd is still unclear (Ernstrom, 1974). It is hoped that the present study can contribute to the elucidation of this mechanism and help to define the relationship between milk clotting and general proteolysis, two closely similar processes.

In the present project, porcine pepsin was modified with limited quantities of reagents to yield limited modification of the carboxyl groups. Extensive derivatization of carboxyl groups is detrimental to pepsin activity since the two active-site amino acids were shown to be aspartic acid (Bayliss et al., 1969; Chen and Tang, 1972).

The activities of the modified pepsin against various substrates including synthetic dipeptides, hemoglobin, reconstituted skimmilk, whole casein and κ - and α_{s1} -caseins, were measured. Some physical properties of the carboxyl modified enzyme such as pH profiles and electrophoretic mobility, and properties pertinent to the performance of pepsin in cheese-making such as pH stability, curd tension and rate of syneresis were studied. Some chemical properties of the modified enzyme such as the response to inhibitors and kinetics were also studied. The characteristics of the modification, including the specificity of the reaction and the effect of substrates on the modification, were investigated.

LITERATURE REVIEW

Chemical Modification of Proteins

In its broadest sense, any transformation which involves the formation or rupture of a covalent bond may be regarded as chemical modification of a protein. This would include processes such as proton transfer, metal chelation, enzyme-substrate interaction and even hydrogen bonding (Cohen, 1970). In a more restricted sense, however, chemical modification is generally referred to as the intentional alteration of protein structure or conformation by chemical agents (Means and Feeney, 1971). Essentially, it involves the derivatization by specific reagents of some reactive side-chain groups in the protein molecule such as charged anionic and cationic groups, hydroxyl, amide and thiol residues.

Chemical modification of proteins has a long history related to the pharmaceutical, dyeing and clothing industries. The treatment of animal hides or hairs for human use as in the tanning of leather is one of the oldest processes utilizing chemical modification. This ancient procedure was recently improved by the use of glutaraldehyde, a cross-linking reagent of proteins. Similarly, several modifications were used to give wool fibres superior performance for clothing. Formaldehyde had been used to

modify bacterial toxins, rendering them incapable of eliciting a toxic response but still retaining its ability to produce an immunological response when injected into an animal.

With the availability of new, specific chemical reagents and more sophisticated analytical techniques, chemical modification has become one of the most powerful tools of protein chemists for the study of structures and functions of biologically-active proteins (Cohen, 1968; Stark, 1970; Means and Feeney, 1971; Knowles, 1974; Glazer et al., 1976). Chemical modifications are routinely used to investigate the roles of individual amino acid chains in relation to the physical, chemical and biological properties of proteins and to determine the active-site residues in enzymes.

Protein modification has wide application in other areas of biochemical research including immunochemistry, X-ray crystallography and purification of proteins. By modification of an antibody and determination of changes in its ability to interact with antigen, information can be obtained on the functional groups involved in the bonding (Singer, 1965; Haber, 1968). Determination of the crystal structure of a protein at high resolution requires the preparation of heavy atom derivatives which are isomorphous with the parent crystal. Heavy atoms may be incorporated

by selective chemical modification which makes important contributions to X-ray crystallography (Benisek and Richards, 1968). Some proteins form reversible complexes with other substances and have properties which can be used to separate proteins by conventional methods. One example is the separation of a group of closely related antifreeze glycoproteins from blood serum of Antarctic fishes by complexing with borate (Vandenheede, 1972).

In the food industry, chemical modification is mainly used to improve functional properties of proteins. Alkali treatments have been used in the solubilization of protein-containing materials in preparation for extrusion processing (Van Beek et al., 1974). Hydrolysates of some proteins have improved flavor characteristics, better emulsifying property and improved foaming ability (Richardson, 1977).

The other chemical approach to modify the functionality of proteins which has received much attention is the use of various acylating agents. Egg white modified with 3,3-dimethyl-glutaric anhydride shows increased heat stability while the foaming capacity is not significantly affected (Gandhi et al., 1968). Vegetable proteins such as soy proteins have been modified with acetic and succinic anhydrides to be used in coffee whitener formulations

(Melnychyn and Stapley, 1973). The acylated proteins have decreased viscosity and isoelectric point as well as mild flavor and odor. Unlike conventional vegetable protein isolates, they do not "feather" or precipitate when added to hot coffee or tea. N-Succinylated egg yolk proteins and ovalbumin have been successfully made for use in mayonnaise and salad dressing (Evans and Irons, 1971a; b). Other acylated proteins including whey, casein, serum and gelatin are also used for stabilizing oil-in-water emulsions and in ice cream mix (Evans, 1970a; b). Fish myofibrillar proteins have been succinylated and found to form viscous aqueous dispersions with good heat stability, a high emulsifying capacity, bland flavor and improved foaming stability (Groninger, 1973; Groninger and Miller, 1975; Chen et al., 1975).

Apart from improving the functional properties, food proteins are modified by chemical methods to block deteriorative reactions and to improve nutritional value. Protein amino groups have been modified by acylation (Bjarnason and Carpenter, 1970) and dimethylation (Galembeck et al., 1977) to block the Maillard reaction. The nutritional quality of food proteins can be improved by increasing the digestibility of the proteins, inactivating toxic or inhibitory substances or attaching essential nutrients to the proteins. Coloring and flavoring agents can also be attached to improve the

acceptability of food proteins (Feeney, 1977).

Modification of Carboxyl Groups

Two reactions most commonly employed to modify the carboxyl groups of proteins are esterification and coupling with nucleophiles mediated by a water-soluble carbodiimide.

1. Esterification

Protein carboxyl groups may be esterified by a number of procedures. Carboxyl groups can be converted to methyl esters in methanol containing small amounts of hydrochloric acid (Wilcox, 1967). Unless done under limiting conditions that may result in incomplete reaction, acid-catalysed esterification can lead to side reactions such as N→O acyl shift or deamidation (Wilcox, 1967; Cohen, 1968). The reaction is also accompanied by conformational changes which preclude its use with most proteins. However, the procedure has been used successfully to study the role of carboxyl groups in more stable proteins such as lysozyme (Fraenkel-Conrat and Olcott, 1945; Frieden, 1956), chymotrypsinogen (Doscher and Wilcox, 1961) and bovine serum albumin (Ram and Maurer, 1959).

Diazo compounds such as derivatives of diazoacetic acid are widely used to esterify carboxylic acids. Diazoacetates react with water and many simple inorganic anions,

limiting modification to the more accessible carboxyl groups. Modification of only 20-30% of the carboxyl group is usually possible with a typical protein. Diazoacetates react optimally with protein carboxyl groups near pH 5. At lower pH's, hydrolysis of reagent becomes appreciable and limits the extent of modification. This procedure has been used to modify pancreatic ribonuclease (Riehm and Scheraga, 1965), chymotrypsinogen A (Doscher and Wilcox, 1961) and pepsin (Rajagopalan et al., 1966a; Lundblad and Stein, 1969).

Alkylhalides are too unspecific for esterification of protein carboxyl groups unless they also behave as an affinity label, as in the esterification of the active-site aspartic acid of pepsin by p-bromophenacyl bromide (Gross and Morell, 1966; Erlanger et al., 1966) and the active-site glutamic acid of ribonuclease T₁ by iodoacetate (Takahashi et al., 1967). Triethyloxonium fluoroborate has been used to esterify specifically aspartic acid 52 in lysozyme (Parsons and Raftery, 1969).

2. Carbodiimide reaction

The most popular method for modifying carboxyl groups in proteins involves the use of water-soluble carbodiimides. The carbodiimides react with carboxyl groups at slightly acidic pH to give an O-acylisourea (Khorana, 1953), an

activated intermediate that can either rearrange to an acylurea or react with a nucleophile as shown in Fig. 1. If the nucleophile is an amine, it will condense with O-acylisourea to yield the corresponding amide.

The carbodiimide reaction has been widely used in the determination of the carboxyl group content of proteins as well as in the study of carboxyl group function. Under mild conditions, only the more accessible or reactive carboxyl groups react, while in the presence of denaturants and excess reagents, nearly quantitative substitution can be obtained (Hoare and Koshland, 1967; Carraway and Koshland, 1972).

This procedure offers considerable flexibility in the choice of carbodiimide and nucleophile. Several water-soluble carbodiimides have been used for protein modification. 1-Cyclohexyl-3-(2-morpholinyl-4-ethyl)-carbodiimide metho-p-toluenesulfonate and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide are commercially available. 1-Benzyl-3-(3-dimethylaminopropyl) carbodiimide is also frequently used (Hoare and Koshland, 1966). All carbodiimides react similarly although the smaller reagents might be expected to be more accessible to partially buried carboxyl groups.

Different nucleophiles can be used to suit particular experimental conditions. The ionic character of proteins

FIGURE 1. THE REACTION OF PROTEIN CARBOXYL GROUP WITH WATER-SOLUBLE CARBODIIMIDE AND NUCLEOPHILE.

Nu : NUCLEOPHILE.

can be varied considerably depending on the charge of the nucleophiles (Fig. 2). Radioactive or colored amines can be employed to facilitate quantitation and identification (Matyash et al., 1973).

Carbodiimide also reacts with both sulfhydryl and tyrosyl-OH groups. Tyrosine can be regenerated by treatment of the derivative with hydroxylamine at pH 7 (Carraway and Koshland, 1968). Successful regeneration of thiol groups has not been reported due to excessive stability of the resulting product (Carraway and Triplett, 1970).

Pepsin

The catalytic activity of pepsin in the digestive function of stomach was first identified by the Italian researcher Abbé Spalanzani in 1783 (cited by Gillespie, 1898). This was perhaps the first scientific demonstration of enzymatic activity. Pepsin was the first enzyme to be named (by T. Schwann in 1825) and the second enzyme to be crystallized (Northrop, 1930).

The term pepsin is applied to the gastric proteinases formed by partial proteolysis of their inactive zymogens, pepsinogens. The nomenclature of pepsin is complicated by the multiplicity of the enzyme. However, pepsin (E.C. 3.4.23.1) has been referred to by most authors and by the

FIGURE 2. DEPENDENCE OF CHARGE ON NUCLEOPHILES ON THE MODIFICATION OF PROTEIN CARBOXYL GROUP.

Enzyme Commission as porcine pepsin A, the major gastric proteinase from pig. Most of the available data on the chemical structure and kinetic properties of pepsin are from studies on porcine pepsin A and its zymogen, pepsinogen A. Highly purified or crystalline pepsin preparations have been obtained from the gastric mucosa of man, cow (Northrop, 1933), chicken (Levchuk and Orekhovich, 1963) and fish (Norris and Elam, 1940; Sprissler, 1942).

Pepsin is a proteinase (or protease) which catalyses the cleavage of peptide bonds in the substrate proteins. Unlike other types of proteases (serine, metallo, and sulfhydryl proteases), pepsin functions optimally in a very acidic medium of about pH 2-3. Pepsin and pepsin-like enzymes are therefore referred to as acid proteases.

The primary structure of porcine pepsin was determined recently (Tang et al., 1973; Sepulveda et al., 1975). It contains 327 amino acid residues. Pepsin structure is unique in that only one lysine and two arginines are found within the terminal 20 residues of the carboxyl end of the protein. The other 307 residues contain only one basic amino acid, histidine-97. Along the same stretch are 44 acidic residues, including a phosphoserine. The three-dimensional structure of porcine pepsin has been resolved at 0.27 nm resolution (N. Andreeva, cited by Tang, 1976).

The active-site residues of pepsin have been identified as Asp-32 (Bayliss et al., 1969) and Asp-215 (Chen and Tang, 1972). They are found to be located in an apparent binding cleft in the three-dimensional structure.

Table 1 summarizes some physical properties of porcine pepsin and pepsinogen.

Pepsinogen, the precursor of pepsin, is activated at pH below 5 to form pepsin upon the removal of the 41-residue amino-terminal portion of the zymogen. The mechanism for pepsinogen activation is still not known, although several models have been proposed (Tang, 1970; Al-Janabi et al., 1972; Kassell and Kay, 1973).

Pepsin is a protease with broad side chain specificity (Hill, 1965). The sensitive bonds are generally present in dipeptidyl units containing at least one hydrophobic amino acid residue such as phenylalanine, tyrosine, leucine and methionine (Tang, 1963). Peptides containing p-nitro-phenylalanine (Inouye and Fruton, 1967), 3,5-dinitrotyrosine (Knowles et al., 1969) and diiodotyrosine (Jackson et al., 1965) are hydrolysed by pepsin and have been used in kinetic investigations.

In addition to hydrolysing peptide bonds, pepsin catalyses transpeptidation of the amino-transfer type (Neumann et al., 1959; Fruton et al., 1961). Pepsin can also act as an esterase, hydrolysing ester linkages in suitable

Table 1. Some physical properties of porcine pepsin and pepsinogen.

	Pepsin	Pepsinogen	Reference
$s_{20, w}$ (sec X 10^{13})	2.96-3.0 ^a	3.2-3.35 ^b	a) Blumenfeld and Perlmann, 1959. b) Arnon and Perlmann, 1963.
$D_{20, w}$ (cm ² /sec X 10^{13})	8.70 ^c	7.54 ^c	c) Orekhovich et al., 1956.
Molecular Weight	35,000 ^a	41,000 ^b	
λ_c (nm)	216 ^d	236 ^e	d) Ryle, 1960. e) Ryle, 1965.
$-\alpha]_{366 \text{ nm}}$	232° ^d	212° ^e	
$E_{278 \text{ nm}}^{\text{Mol}}$	50,990 ^f (MW=35000)	51,300 ^f (MW=41000)	f) Ryle and Porter, 1959.

substrates (Lokshina et al., 1964; Inouye and Fruton, 1967).

Pepsin has been demonstrated to participate in the "plastein reaction" (Wasteneys and Borsook, 1930). It catalyses peptide bond synthesis by direct condensation of α -amino and α -carboxyl groups of oligopeptides at high substrate concentration (Determann et al., 1965).

Pepsin belongs to a group of enzymes closely similar in structure and activity. These include other gastric enzymes (e.g., gastricsin and chymosin), proteases from microorganisms, proteases from plants, mammalian lysosomal proteases (e.g., cathepsin D and E) and proteases in blood plasma and seminal plasma. The amino acid sequences of all the acid proteases studied so far are homologous (Sepulveda et al., 1975), apparently a result of divergent evolution from a common ancestral protein (Hofmann, 1974; Tang, 1976). The active sites of the acid proteases are also alike and these enzymes are inhibited by the same site-specific inhibitors. These facts suggest that enzymes in this group have a similar catalytic mechanism, although their specificity may vary due to differences in the topography of their binding sites (Tang, 1976).

Chemical Modification of Pepsin

Chemical modification has contributed greatly to the identification of active-site residues in pepsin as well as other amino acid side groups essential for pepsin activity.

Diazoacetyl norleucine methyl ester, a derivative of diazoacetic acid, was found to inactivate pepsin by esterification of only one carboxyl group (Rajagopalan et al., 1966a; Lundblad and Stein, 1969). Isolation and sequence analysis of a peptide containing the diazo modified residue indicates that the esterified aspartyl group is located at residue 215 (Bayliss et al., 1969).

A second carboxyl group in the active site of pepsin was determined by the use of a substrate-like epoxide in-activator, 1,2-epoxy-3-(p-nitrophenoxy) propane (EPNP). Sequence determination of the EPNP-containing peptide placed the aspartyl residue at position 32 (Chen and Tang, 1972).

A third carboxyl group in pepsin was modified by bromophenacyl bromide (Erlanger et al., 1965). However, the fact that the fully reacted enzyme retained about 20% of its activity and that other acid proteases were unaffected suggests that this modified group was not directly involved in catalysis (Clement, 1973).

The presence of an arginyl residue near the active centre of pepsin was demonstrated by modification with phenylglyoxal (Kitson and Knowles, 1971) and butanedione

(Huang and Tang, 1972), and was found to be Arg-316. The fully reacted enzyme retained about 20% of activity. The reaction was retarded by the presence of peptide substrates indicating the proximity of the modified residue to the active centre of the enzyme (Huang and Tang, 1972).

Selective modification of carboxyl groups in pepsin was carried out using a colored amine, N-(2,4-dinitrophenyl)-hexamethylenediamine and a water-soluble carbodiimide (Matyash et al., 1973). Incorporation of one amine molecule per molecule of pepsin led to a drop of activity of about 40%. Three carboxyl groups were modified: β -carboxyl group of aspartate, γ -carboxyl group of glutamate and the α -carboxyl group of C-terminal alanine.

N-Bromosuccinimide has been used to modify tryptophan residues in pepsin (Lokshina and Orekhovich, 1964; Green and Witkop, 1964). Pepsin inactivation by the reagent amounted to 85-90%. Dopheide and Jones (1968) used 2-hydroxyl-5-nitrobenzyl bromide for tryptophan modification. Incorporation of two residues of the reagent was observed, resulting in a loss of only 25-30% of proteolytic and peptidase activity.

Methionine residues in pepsin were alkylated by iodoacetic acid with no observable change in activity, indicating that methionine residues were not important for the action of pepsin (Lokshina and Orekhovich, 1964). The amino groups

of pepsin were also shown to be non-essential for enzyme activity. Acetylation of the amino groups with ketene did not affect protease activity (Herriott and Northrop, 1934). This was further supported by deamination with nitrous acid (Philpot and Small, 1938) and N-ethoxyformylation (Melchior and Fahrney, 1970) both of which caused no appreciable change in activity.

Tyrosine residues have been modified by acetylation and iodination. A 95% inhibition in proteolytic activity was observed after acetylation of 11-12 tyrosine-OH groups in the pepsin molecule. The esterase and peptidase activity, however, was increased (Lokshina and Orekhovich, 1966). In comparison, iodination led to a decrease in protease, peptidase and esterase activities (Holland and Fruton, 1968). Sequence analysis of the iodinated peptides located the modified tyrosine residues at positions 9 and 175 (Mains et al., 1973).

MATERIALS AND METHODS

Biochemicals and Special Chemicals

All chemicals used were of reagent grade unless specified otherwise.

Porcine pepsin (E.C. 3.4.23.1; from hog stomach mucosa, 2X crystallized) was purchased from Sigma Chemical Co. and Worthington Biochemical Corp. The lot from Sigma had an assay of 3,520 units/mg protein, while the lot from Worthington had an assay of 2,700 units/mg protein. Crude pepsin (1:10,000), pepsinogen (from hog stomach) and chymosin (rennin, E.C. 3.4.23.4; from calf stomach) were products of Sigma Chemical Co. Rennet (NF rennin) was purchased from ICN Pharmaceuticals, Inc.

The two water-soluble carbodiimides used in this project, 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide metho-p-toluenesulfonate and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide were purchased from Sigma Chemical Co. Glycine methyl ester hydrochloride was a product of Aldrich Chemical Co., Inc., while the methyl esters of arginine, leucine, lysine, tyrosine and tryptophan were from Sigma Chemical Co. Ethylenediamine dihydrochloride and taurine (2-aminoethanesulfonic acid) were also from Sigma Chemical Co.

The synthetic dipeptides, N-acetyl-L-phenylalanyl-diiodo-L-tyrosine, N-acetyl-D-phenylalanyl-L-tyrosine and N-carbobenzoxy-L-glutamyl-L-tyrosine were products of Sigma Chemical Co. Two pepsin inhibitors, 1,2-epoxy-3-(p-nitrophenoxy) propane and bromophenacyl bromide were purchased from Eastman Kodak Co. The substrate for the determination of proteolytic activity, acid-denatured hemoglobin (bovine serum), was from Worthington Biochemical Corp.

3-(2-Aminoethyl) indole and p-toluenesulfonic acid were products of Matheson Coleman & Bell, Norwood, Ohio. Ninhydrin and hydrindantin were purchased from Eastman Kodak Co.

Methods

1. Modification of carboxyl groups

The carboxyl groups in pepsin, pepsinogen and chymosin were modified by the carbodiimide-promoted amide formation. The method was essentially that described by Hoare and Koshland (1967) for the determination of carboxyl content in proteins. However, sufficiently lower concentrations of carbodiimide and nucleophile were used to achieve selective modification. Denaturants such as 8 M urea or 6 M guanidine hydrochloride, as suggested by the above workers for total covering of carboxyl groups, were omitted in the present investigation.

The protein (10 mg/ml) and nucleophile were dissolved in distilled water. The pH of the solution was adjusted with 1 N NaOH to 5.5. The water-soluble carbodiimide was added as a solid to bring its concentration to the desired level. The reaction mixture was kept at room temperature and stirred continuously for 90 minutes. The pH of the reaction mixture was kept constant at 5.5 by automatic addition of 0.1 N HCl, using a Radiometer type TTTic titrator (Copenhagen, Denmark). The reaction was terminated by the addition of excess 3 M sodium acetate buffer at pH 5.5 which reacted with excess nucleophile. The residual reagents were removed by exhaustive dialysis against dis-

tilled water for 48 hours at 4° C and the modified protein was recovered by lyophilization.

To determine the extent of carboxyl modification, the modified protein was subjected to amino acid analysis. The protein was hydrolysed for 24 hours at 110° C with 3-(2-aminoethyl) indole and p-toluenesulfonic acid, as described by Liu and Chang (1971). The hydrolysate was then neutralized and applied to an amino acid analyser column. From the chromatogram, the peak area of the particular amino acid which had been incorporated was measured and compared to that of the control. The increase in peak area was proportional to the number of molecules of amino acid methyl ester incorporated into the protein, or the number of carboxyl groups modified.

2. Determination of milk clotting activity

The milk clotting activity of pepsin and chymosin was determined by the methods of Berridge (1945) and Foltmann (1970) with some modifications. Commercial spray-dried skimmilk powder was used as substrate and was kept desiccated at 4° C. The substrate solution was prepared by reconstituting 12 g of skimmilk powder in 100 ml of 0.01 M CaCl_2 with vigorous stirring for 5 minutes without foaming. The reconstituted skimmilk had a pH of about

6.3. The pH can be adjusted by varying the concentration of CaCl_2 from 0.001 M (pH=6.5) to 0.08 M (pH=5.7) and the addition of small amount of HCl or NaOH.

The reconstituted skimmilk substrate was left at room temperature for one hour to equilibrate. The skim-milk (10 ml) was pipetted into a stoppered test tube and incubated in a water bath at 30°C for 10 minutes. The enzyme was diluted with citrate buffer to an activity corresponding to a clotting time of 4-5 minutes. The diluted enzyme solution (1 ml), pre-incubated to 30°C , was pipetted into the test tube with thorough mixing. A stopwatch was started. The milk was kept flowing from one end of the stoppered tube to the other. The moment when the thin film of milk broke into visible particles was recorded as the clotting time.

As defined by Berridge (1945), one unit of milk clotting activity was the amount of enzyme which would clot 10 ml of reconstituted skimmilk in 100 seconds at 30°C . The specific activity was expressed as milk clotting activity per mg protein. The enzyme concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as a protein standard.

3. Determination of proteolytic activity

The assay method of Anson (1938) was followed with modifications. Acid-denatured hemoglobin was used as substrate. Substrate solution was freshly prepared by dissolving hemoglobin powder in distilled water. Hydrochloric acid was added to yield the desired pH, and the concentration was adjusted to 2.0% (w/v) protein by the addition of distilled water.

The enzyme (0.5 ml), in suitable dilution, was added to a stoppered test tube containing 0.5 ml of 0.1 M KCl/HCl buffer (citrate buffer for pH's above 2.5) of appropriate pH. For routine analysis, the optimum pH for pepsin activity (pH 2.0) was used. The enzyme and substrate solutions were equilibrated in a water bath at 37°C for 10 minutes. The substrate solution (0.5 ml) was added to the assay tubes with vigorous mixing using a vortex mixer. After incubation at 37°C for exactly 10 minutes, 10 ml of 5% (w/v) trichloroacetic acid (TCA) were added to the assay tubes. TCA stopped the enzyme reaction and precipitated the unhydrolysed substrate. The samples were filtered through Whatman No. 2 papers and the absorbance at 280 nm of the filtrate was read with a Beckman DB spectrophotometer using 1 cm cuvettes. A blank was run in which TCA was added before the enzyme solution. The absorbance of the blank was measured and deducted from the sample absorbance. All

determinations were run in duplicate and the averages were used for calculating the proteolytic activity.

One unit of proteolytic activity was defined as the amount of enzyme that produced an absorbance of 0.001 per minute at 37° C. The specific activity was expressed as the proteolytic activity per mg protein.

4. Determination of peptidase activity

The peptidase activity of pepsin was determined using N-acetyl-L-phenylalanyl-diiodo-L-tyrosine (APDT) as substrate. The assay method of Jackson et al. (1965) was followed with some modifications as described by Ryle (1970).

Ninhydrin reagent was prepared by dissolving 20 g ninhydrin and 6 g hydrindantin in 750 ml of 2-methoxyethanol. To this was added 250 ml of acetate buffer (544 g $\text{CH}_3\text{COO} \cdot 3\text{H}_2\text{O}$ + 100 ml of glacial acetic acid). The reagent was stored under nitrogen in a dark bottle equipped with a dispenser. The substrate solution (1.0 mM), prepared by dissolving APDT in 0.01 N NaOH, was stable for several weeks when kept in the cold room.

The enzyme solution (0.5 ml) was added to tubes with 0.25 ml of HCl of appropriate normality and incubated in a water bath at 37° C. After 10 minutes, 0.25 ml of APDT solution was added to the reaction tubes and 1.0 ml of

ninhydrin reagent was added to the blanks. After exactly 10 minutes, 1.0 ml of ninhydrin reagent was pipetted to the reaction tubes and at any time 0.25 ml of APDT was added to the blanks.

All the tubes, stoppered by marbles, were placed in a boiling water bath for 15 minutes to develop the color, and were then cooled with cold water. The contents of the tubes were diluted with 5 ml of 60% (v/v) ethanol. After mixing, the absorbance of the solution at 570 nm was read against the blanks. Duplicates were run for all samples and blanks, and the averages were used to calculate the peptidase activity, expressed as APDT units.

One APDT unit is defined as the quantity of enzyme which liberates 1.0 micromole of diiodotyrosine per minute at 37° C. Since E_{mmol} for the color reaction of diiodotyrosine is 22.8, the volume of the solution was 7 ml and the time of incubation was 10 minutes, $A_{570 \text{ nm}}$ was converted to APDT units by multiplying by 0.0306. Specific peptidase activity was expressed as APDT units/mg protein.

5. Assay of pepsin with κ -casein and α_{s1} -casein

Pepsin activity was assayed by a method using κ -casein as substrate (Douillard and Ribadeau-Dumas, 1970). κ - α_{s1} -Casein mixtures were also used as substrate.

κ -Casein and α_{s1} -casein were prepared by the method of Zittle and Custer (1963). Solutions of κ -casein and α_{s1} -casein (0.2% w/v) were made by dissolving the proteins in 0.05 M citrate buffer, pH 5.3. A mixture of κ -casein and α_{s1} -casein (1:1) was prepared by mixing equal volumes of the 0.2% solutions, which gave a final concentration of 0.1% κ -casein and 0.1% α_{s1} -casein. The substrate solutions were incubated in stoppered test tubes at 37° C. Enzyme solution of appropriate concentration was added with immediate mixing. The time at which visible particles appeared was taken as the clotting time. One unit of activity was arbitrarily defined as the amount of enzyme which would clot 10 ml of casein solution in 100 seconds at 37° C. Specific activity was expressed as clotting activity per mg protein.

6. Determination of rate of casein hydrolysis

The rate of release of non-protein nitrogen from whole casein by pepsin was determined by a method described by Green (1972). The substrate solution contained 1% (w/v) whole casein in 0.1 M sodium citrate buffer, pH 5.3 or 6.5. After incubation at 30° C for 10 minutes, enzyme was added. At time intervals, samples were withdrawn and sufficient 10% (w/v) TCA was added to give a final TCA concentration

of 3%. After filtering through Whatman No. 2 papers, the nitrogen content of the filtrate was determined by the procedure of Lang (1958). The rate of liberation of TCA-soluble nitrogen was expressed as $\mu\text{g N released/mg casein}$.

7. Agarose gel electrophoresis

Electrophoresis of enzymes and milk proteins was carried out with the agarose film cassette system of Analytical Chemists, Inc. (Palo Alto, California)

The agarose films contained 1.2% agarose, 10% sucrose and 0.035% EDTA in 0.05 M barbital buffer, pH 8.6. The plate consisted of a thin layer of agarose gel adhered to a transparent plastic backing. Samples (1-2 μl) containing 20-40 μg proteins were pipetted into the pre-cast sample wells with a Hamilton microliter sample dispenser and a disposable tip. The agarose plate was fitted into a cassette cover. Electrophoresis was started by inserting the film-loaded cassette onto the electrophoresis cell / power supply unit. The electrophoresis cells contained 200 ml of 0.05 M barbital buffer, pH 8.6, with 0.035% EDTA. Since the film carried a voltage gradient of 15 V/cm with negligible heat buildup, no cooling was necessary.

After electrophoresis which took 35-50 minutes, the plate was removed from the cassette cover and stained with

0.2% (w/v) amido black 10B in 5% (v/v) acetic acid for 15 minutes in a staining bath with continuous stirring by a magnetic stirrer. The stained film was rinsed with 5% acetic acid and dried at 72° C for 15 minutes in an oven. The dried film was destained by washing twice with 5% acetic acid, and the destained gel plate was dried again in the oven.

For electrophoresis of milk curds, the curds were dissolved in the electrophoretic buffer containing 6 M urea. The agarose film was equilibrated for one minute with the urea-containing barbital buffer before sample application.

8. Determination of isoelectric point

The isoelectric point of pepsin samples was determined by gel electrophoresis at different pH's. The agarose film was cut up into strips and equilibrated in 0.1 M buffer of appropriate pH (KCl/HCl buffer for pH 1-2, citrate buffer for pH 3-5). After sample application, each strip was connected by buffer contained in two 5-ml beakers which were placed in the two electrode compartments. Each compartment was filled with sufficient 0.2 M NaCl solution which was connected to the buffer in the beakers by a small paper strip. Five samples were run at the same time in buffers with pH's of 1.0, 2.0, 3.0, 4.0 and 5.0.

After electrophoresis, the pH's of the buffers were measured to detect pH change that may occur during the run. After staining, the mobilities of the enzyme bands were measured and plotted against the pH's. The straight line curve obtained by linear regression analysis was extrapolated to cut the pH axis which corresponded to zero mobility. This was the estimated isoelectric point of the enzyme.

9. Determination of K_m and k_{cat}

The initial velocity (v_o) of the pepsin-catalysed hydrolysis of APDT, expressed as μ moles diiodotyrosine liberated per minute, was measured at different substrate concentrations. The Michaelis constant, K_m , was determined from the Lineweaver-Burk plot which is the reciprocal of substrate concentration against the reciprocal of v_o . Linear regression analysis was employed to yield the best-fit curves. The molecular activity coefficient or catalytic constant, k_{cat} , was calculated by the Michaelis-Menten equation:

$$v_o = k_{cat} [S][E] / (K_m + [S])$$

where $[E]$ was the total enzyme concentration and $[S]$ was the substrate concentration at zero time. The concentration of pepsin was estimated from the absorbance at 280 nm, assuming a molar absorptivity of 50,990.

K_m and k_{cat} were determined at both pH 2.0 and 4.5.

10. Assay of pepsinogen

The proteolytic and milk clotting activities of porcine pepsinogen were determined after activation of the zymogen. Pepsinogen was dissolved in 0.01 M KCl/HCl buffer, pH 2.0 at a concentration of 1 mg/ml. The solution was kept at room temperature overnight when all the zymogen was converted to the active enzyme (Herriott, 1938).

11. Determination of pepsin stability near neutral pH

The pH stability of both crystalline and crude pepsins was determined by the methods described by Lowenstein (1974) and Green (1972).

The enzyme stability in buffer was measured by incubation of a suitable concentration of pepsin in 0.05 M phosphate buffer, pH 6.5, at 30° C. At time intervals, an aliquot of sample was removed and the milk clotting and proteolytic activities were determined. The changes in activities were followed for 2-4 hours.

The stability of pepsin in milk ultrafiltrates was also studied. Reconstituted milk (from skimmilk powder) was passed through an ultrafiltration cell (Model 52, Amicon Corp., Lexington, MA.) equipped with a PM Diaflo ultrafilter. The filtrate collected was stored at 4° C and used within two days after preparation.

The enzyme was incubated at 30° C in the milk ultrafiltrate which has been previously adjusted to pH 6.60 with 1 N lactic acid. The pH of the incubation mixture was lowered by adding lactic acid at 15-minute intervals, at approximately the same rate at which it was lowered during cheese-making. The scheme suggested by Green (1972) was followed (Table 2). Samples of enzyme were taken at various time intervals for assay of milk clotting and proteolytic activities.

12. Determination of thermal stability of pepsin

The thermal stability of pepsin was determined by measuring both milk clotting and proteolytic activities of the enzyme at increasing temperatures. For proteolysis, the activity was measured at 30, 40, 50, 60 and 70° C. For milk clotting, the activity was determined at 25, 30, 35, 40, 45 and 50° C. The pH was 2.0 for proteolysis and 6.3 for milk coagulation. The Q_{10} values were calculated which represent the ratio of the activity at temperature $(T + 10)^{\circ}$ to that at T° .

Table 2. Adjustment of pH of milk ultrafiltrate for stability test of pepsin.

Incubation Time (minute)	pH	Corresponding Cheese-making Stage
1	6.60	Enzyme addition
50	6.50	Cutting
120	6.40	Maximum scald
160	6.25	-
210	5.90	Pitching

13. Determination of curd tension

The firmness or curd tension of the coagulum from pepsin-curdled milk was determined by a method developed by Hehir (1968), later improved by Ellis (1972). This technique involves the measurement of the weight transfer when a weight is placed on the surface of a coagulated milk sample.

Plastic beakers (50 ml) were used for curd tension determinations and were reduced to a standard weight by shaving the rims. Using the tare facility on a 1,200 g (± 0.01 g) capacity, digital top-pan balance (Sartorius 3704) the weight of the beakers was reduced to zero.

A standard volume of reconstituted skim milk (19.8 ml) was pipetted into a tared beaker. The beaker was placed in a water bath at 30°C . After incubation for 5 minutes, 0.2 ml of appropriately diluted enzyme was added to the milk with mixing. The concentration of enzyme was such that milk was clotted in 4-5 minutes (T). The coagulated milk sample was incubated at 30°C for further 30 minutes which is a convenient time to give curd formation.

At the precise time (T + 30 minutes), the beaker of coagulum was gently taken from the water bath, dried on the outside with tissue paper and placed on the balance pan. Exactly 15 seconds after removal from the bath, a flat bottomed 10 g weight supported by a metal chain was

placed on the surface of the curd. The scale was read to the nearest 10 mg (centigram) at exactly 15 seconds (to minimize surface tension effect) and 60 seconds after the weight was in contact with the curd, i.e., 30.5 minutes and 31.25 minutes after curd formation. The difference between the two readings, in centigrams, was taken as an arbitrary measure of the curd tension which was the amount of weight transferred from the curd to the chain in 45 seconds.

A constant displacement of the hanging weight was set by placing 20 ml distilled water in a standardized beaker. By adjusting the height of the point of support of the weight, the balance was set arbitrarily at 20.70 g when about 70% of the weight's volume was immersed. This operation ensured a constant distance between the point of chain suspension and the curd surface.

14. Determination of the rate of syneresis

The rate of exudation of whey from curd was measured by the discontinuous method of Lawrence (1959) with some modifications.

Samples of reconstituted skim milk (100 ml) at pH 6.3 were added to 250-ml beakers and incubated at 30°C in a water bath. An appropriate amount of enzyme was added to the

milk. The gel was cut into blocks of uniform size 30 minutes after clotting. The curd was then held at 30° C without stirring.

At different time intervals, each beaker was tipped on to a sieve placed over a funnel. The curd was retained by the sieve and the whey was drained into a graduated cylinder under the funnel. The volume of whey in the cylinder 30 seconds after tipping was recorded. Volume of the curd was calculated by subtracting the volume of the whey from the initial volume of milk. The result was expressed as percentage syneresis:

$$\text{Percentage syneresis} = \frac{\text{volume of whey}}{\text{initial volume of milk}} \times 100$$

RESULTS AND DISCUSSION

Choice of Enzyme Source

The pepsin samples used in the present investigation were 2X crystallized and lyophilized enzyme from hog stomach mucosa. Two lots, one from Sigma Chemical Co. (Lot No. 67C-8195) and the other from Worthington Biochemical Corp. (Lot No. PM 37C675), were used. Both enzymes appeared as one sharp band when applied to agarose gel electrophoresis, and were eluted as one sharp peak on DEAE cellulose column with 0.1 M sodium acetate buffer (pH 4.2) and increasing concentration of NaCl as eluents. These observations suggest that the enzyme preparations used were essentially homogeneous and were therefore used directly without further purification.

Rajagopalan et al. (1966b) proposed a method to prepare homogeneous pepsin from pepsinogen. The procedure involved activation of the zymogen at 14°C and pH 2 for 20 minutes and the separation of the enzyme from the peptide by passage through a column of sulfoethyl Sephadex C-25. The high cost of pepsinogen prohibited preparation of large amount of pepsin by this method.

In some experiments, crude pepsin (1:10,000) was used. The industrial pepsin is the starting material from which crystalline pepsin has traditionally been prepared.

This is the enzyme preparation that can be used together with calf rennet as 50:50 mixtures to make cheese. Hence, in experiments designed to test the suitability of modified pepsin for cheese-making, 1:10,000 pepsin is a more appropriate enzyme than the crystalline pepsin.

In this thesis, the term pepsin refers to the crystalline porcine pepsin while the impure enzyme will be specified as crude or 1:10,000 pepsin.

Choice of Carbodiimides and Nucleophiles

Two commercially available water-soluble carbodiimides, 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide metho-p-toluenesulfonate (CMC) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were used to modify the carboxyl groups in pepsin. Both carbodiimides were found to modify pepsin carboxyl groups with similar changes in enzymatic activities and properties. However, EDC was found to be more effective than CMC at the same molar concentration. This could be due to the smaller size of EDC (M.W.=191.7) than CMC (M.W.=423.6) enabling EDC to react with partially buried carboxyl groups in pepsin. EDC was therefore used subsequently.

Three nucleophiles, ethylenediamine, methyl esters of various amino acids and 2-aminoethanesulfonic acid

(taurine) were used for carboxyl modification. The different charges carried by these nucleophiles caused considerable difference in the ionic character of the modified enzymes (see Fig. 2). Ethylenediamine and taurine were found to cause extensive loss in milk clotting activity of pepsin even at low concentrations (Table 3). Hence, methyl esters of various amino acids, mainly glycine, were used in later experiments.

Effect of Nucleophile Concentration on the Extent of Carboxyl Modification

The number of pepsin carboxyl groups covered in the carbodiimide reaction was determined by amino acid analysis. EDC and glycine methyl ester at various concentrations were used. The number of carboxyl groups modified increased as the concentration of nucleophile was increased (Fig. 3). The concentration of EDC was found to be less critical in affecting the amount of modification. However, EDC concentrations lower than 10 mM (33-fold excess to pepsin) were found to be ineffective in modifying pepsin.

For most of the subsequent experiments, unless specified otherwise, the concentrations of reagents used were: 33-fold excess of EDC and 174-fold excess of glycine methyl

Table 3. Carboxyl modification of pepsin with different nucleophiles.*

Nucleophile	Concn. (mM)	Proteolytic activity (% control)	Milk clotting activity (% control)
Gly methyl ester	50	94	17
Ethylene- diamine	50	85	3
Taurine	50	90	5

* Pepsin (10 mg/ml) was modified with 33-fold excess of EDC and 174-fold excess of nucleophile at pH 5.5 for 90 minutes.

FIGURE 3. EFFECT OF NUCLEOPHILE CONCENTRATION ON THE EXTENT OF CARBOXYL MODIFICATION OF PEPSIN.

ester, corresponding to an incorporation of 5.2 moles of nucleophile per mole pepsin.

Activity of Native and Carboxyl Modified Pepsins

1. Milk clotting activity

The effect of carboxyl modification on milk clotting activity of porcine pepsin is shown in Fig. 4. The activity was found to drop rapidly with increase in the extent of carboxyl modification. A drop of 70-90% in milk clotting activity was observed.

When treated with glycine methyl ester alone, pepsin retained 100% milk clotting activity. When the carbodiimide concentration was lowered to 5 mM (the nucleophile concentration was 50 mM), the modified enzyme had about 60% of milk clotting activity with a corresponding decrease in the number of modified carboxyl groups (see Table 4). When pepsin was treated with 10 mM EDC in the absence of nucleophile, the milk clotting activity of pepsin was also decreased by about 35% (see Table 4).

When crude pepsin was modified with a low concentration of glycine methyl ester (87-fold excess to pepsin) and EDC (33-fold excess to pepsin), there was a drop of about 50% in milk clotting activity. With a higher nucleo-

FIGURE 4. EFFECT OF CARBOXYL MODIFICATION OF PEPSIN ON MILK CLOTTING ACTIVITY.

Table 4. Effect of carboxyl modification on pepsin activities.

<u>EDC</u>		<u>Gly methyl ester</u>		No. COOH gp. modified/mole pepsin	Milk clotting activity (% control)	<u>Proteolytic activity (% control)</u>	
Concn. (mM)	Excess to pepsin (-fold)	Concn. (mM)	Excess to pepsin (-fold)			pH 2.0	pH 3.5
10	33	0	0	0	65	100	100
10	33	25	87	2.8	27	88	210
10	33	50	174	5.2	17	94	340
10	33	100	348	8.6	13	105	330
10	33	200	706	11.2	9	90	340
0	0	50	174	0	100	100	100
5	16.5	50	174	1.0	60	100	120

phile concentration (174-fold excess to pepsin), the drop in activity was 70%. This indicates that compared to crystalline pepsin, crude pepsin retained higher milk clotting activity when modified with the same quantity of reagents.

Amino acid analysis of the native and modified crude pepsin did not yield consistent results, probably due to impurities present in the enzymes. Hence, the extent of carboxyl modification was not determined for the crude pepsin.

2. Proteolytic activity

The proteolytic activity of pepsin at pH 2.0 was found to decrease slightly after carboxyl modification (Fig. 5). Even with extensive modification (11.2 moles of carboxyl groups covered/mole pepsin), the enzyme still retained 90% of its activity, showing that the carboxyl groups blocked were not directly involved in proteolysis of hemoglobin.

In contrast to the present result, carboxyl modification of porcine pepsin with CMC and a colored amine caused about 40% drop in activity against hemoglobin, although only one amine molecule was incorporated per molecule of pepsin (Matyash et al., 1973). The carboxyl groups modified by the colored amine could be more directly related to pepsin catalysis.

FIGURE 5. EFFECT OF CARBOXYL MODIFICATION OF PEPSIN ON PROTEOLYTIC ACTIVITY.

●, pH 2.0; ○, pH 3.5.

When the activity against hemoglobin was measured at pH 3.5, the carboxyl modified pepsin was found to have activity much higher than the control (Fig. 5). This was mainly due to the fact that at pH 3.5, the control had specific proteolytic activity much lower than that at pH 2.0, while the modified enzyme had higher activity at pH 3.5. This indicates that there may be a shift in the pH profile.

Similar results were obtained when crude pepsin was modified with 33-fold excess of EDC and 87-fold excess of glycine methyl ester. The modified enzyme retained about 90% of its proteolytic activity at pH 2.0, while at pH 3.5, the activity of the modified enzyme was about 2.5 times that of the control.

Table 4 summarizes the effect of carboxyl modification of pepsin on milk clotting and proteolytic activity in relation to the extent of modification.

3. Milk clotting:proteolytic activity ratio

With a marked decrease in milk clotting activity and no significant changes in proteolytic activity, carboxyl modification of pepsin resulted in a dramatic decrease in milk clotting:proteolytic activity ratio (Fig. 6). With extensive modification (11.2 carboxyl groups modified/mole pepsin), the ratio was only 10% that of the control.

FIGURE 6. EFFECT OF CARBOXYL MODIFICATION OF PEPSIN ON MILK CLOTTING:PROTEOLYTIC ACTIVITY RATIO.

The milk clotting:proteolytic activity ratio is an indication of the specificity of proteases. Chymosin, the major enzyme component of calf rennet, was found to have the highest clotting to proteolytic activity ratio among other proteolytic enzymes (Ernstrom, 1974), indicating that chymosin has the highest specific milk clotting activity. The present data show that relative to general proteolysis, the specific activity of pepsin towards milk clotting was lowered after carboxyl modification.

4. Peptidase activity

The peptidase activity of pepsin was measured using APDT as substrate. Result shows that the peptidase activity at pH 2.0 and 37° C was decreased by carboxyl modification (Fig. 7). Unlike milk clotting, however, the decrease in peptidase activity was moderate. The modified pepsin retained about 40-60% of activity against the dipeptide substrate.

Pepsin modified with CMC and a colored amine also showed a drop of 50% in peptidase activity, when N-acetyl-L-phenylalanyl-L-tyrosine was used as substrate (Matyash et al., 1973).

FIGURE 7. EFFECT OF CARBOXYL MODIFICATION OF PEPSIN ON PEPTIDASE ACTIVITY.

Effect of Carboxyl Modification on pH Profiles

1. Milk clotting pH profile

The pH profiles of milk clotting for native and carboxyl modified pepsins are shown in Fig. 8. For both enzymes, the milk clotting activity dropped rapidly from pH 6.0 to 6.5. The modified enzyme retained slightly higher activity at pH 6.3 and 6.5 than the control.

Crude pepsin and the corresponding modified enzyme also showed similar pH profiles, indicating that carboxyl modification did not change the pH profile of milk clotting in pepsin.

2. Proteolytic pH profile

The effect of carboxyl modification on the proteolytic pH profile of pepsin is illustrated in Fig. 9. Native pepsin had a pH optimum at about 2.0 and the specific activity against hemoglobin decreased sharply towards higher pH's. After carboxyl modification, the pH optimum was shifted to about 3.5. The plateau observed at pH 1.5-2.0 for the modified enzyme could be attributed to incomplete modification.

It is interesting to note that chymosin had a proteolytic pH-activity curve closely resembling that of the modified pepsin, with a pH optimum near 3.5 (Fig. 9).

FIGURE 8. MILK CLOTTING pH PROFILES OF NATIVE AND CARBOXYL MODIFIED PEPSINS.

●, NATIVE PEPSIN; ○, CARBOXYL MODIFIED PEPSIN

FIGURE 9. PROTEOLYTIC pH PROFILES OF CRYSTALLINE PEPSIN ●, CARBOXYL MODIFIED CRYSTALLINE PEPSIN ○, AND CHYMOSIN ▲.

A similar shift in proteolytic pH profiles was also observed when crude pepsin was modified with EDC and glycine methyl ester (Fig. 10). The native enzyme had a pH optimum at about 2.0, and after modification the optimum shifted to between pH 3.5-4.0. The modified crude pepsin had a wider pH optimum than the modified pure enzyme.

A shift in pH profile had been noted in some immobilized enzymes and was directly related to the "micro-environment" effect resulting from the embedment of enzymes within the carriers (Silman and Katchalski, 1968). Binding of charged carriers to enzymes may also produce changes in the distribution of charges on the enzyme molecules. Consequently, the pH in the domain of the enzymes will be different from the external bulk solution, thus creating an apparent shift in pH profile (Goldstein, 1970). Since the nucleophile (glycine methyl ester) attached to pepsin was of small molecular size when compared to the carriers used in enzyme immobilization, the shift in pH profile in the modified pepsin was unlikely due to "micro-environment" effect. It was probably related to changes in the charge distribution on the enzyme as a result of covering of the negatively-charged carboxyl groups by the neutral nucleophile.

FIGURE 10. PROTEOLYTIC pH PROFILES OF CRUDE NATIVE AND CARBOXYL MODIFIED PEPSINS.

●, NATIVE PEPSIN (1:10,000);

○, CARBOXYL MODIFIED PEPSIN (1:10,000)

3. pH-Activity curves for the hydrolysis of APDT

When the synthetic dipeptide, APDT, was used as substrate, the pH-activity curves of both native and carboxyl modified pepsin were found to be similar except that the modified pepsin had slightly higher activity at more alkaline pH (Fig. 11). Both enzymes had a pH optimum around 2.0 and the activity decreased rapidly towards higher pH. However, the decline in activity was more gradual in the modified enzyme.

The result shows that unlike hemoglobin, the pH-activity curve on dipeptide substrate was not significantly shifted by carboxyl modification.

The pH-activity curves for APDT hydrolysis were not determined for the crude pepsins.

Electrophoretic Mobility of Native and Carboxyl Modified Pepsins on Agarose Gel

Agarose gel electrophoresis was used to measure the electrophoretic mobility of native pepsin and the carboxyl modified enzymes. Both native and modified enzymes appeared as one band on the agarose film (Plate I). Heterogeneity of chemically modified proteins, resulting from substitution of different numbers of carboxyl groups, might be observed in electrophoresis as a diffused band. The

FIGURE 11. pH ACTIVITY CURVES FOR THE ACTION OF NATIVE AND CARBOXYL MODIFIED PEPSINS ON N-ACETYL-L-PHENYLALANYL-DIIODO-L-TYROSINE.

▲, NATIVE PEPSIN;

△, CARBOXYL MODIFIED PEPSIN.

PLATE I. AGAROSE GEL ELECTROPHORETIC PATTERNS
OF NATIVE AND CARBOXYL MODIFIED
PEPSINS.

NUMBER OF CARBOXYL GROUPS MODIFIED/MOLE ENZYME:

1, 0 (CONTROL);

2, 5.2;

3, 8.7;

4, 11.2;

5, 15.6.

sharpness of the enzyme bands shown in the present result suggest that the derivatives were practically free of unmodified pepsin, and the modified products were essentially homogeneous.

The electrophoretic mobility of pepsin was found to decrease progressively with increases in the extent of carboxyl modification (Plate I).

Electrophoretic separation of proteins on gel matrices such as starch and polyacrylamide is based on both electric charge and molecular size difference of the proteins. However, owing to the low gel concentration and large pore size of the agarose gel used, the molecular sieving effect is minimal. Furthermore, as glycine methyl ester has a molecular weight of 125, the incorporation of ten molecules of nucleophile would increase the molecular weight of pepsin by less than 5%. Gel filtration chromatography of the most extensively modified pepsin (11.2 carboxyl groups modified/mole pepsin) on Sephadex G-200 (Superfine) confirmed that the change in molecular size was not marked, as the modified enzyme was eluted at the same elution volume as the native pepsin (0.1 M acetate buffer, pH 4.5). This result also indicates that there was no aggregation between the modified pepsin molecules. Hence, the drop in electrophoretic mobility after carboxyl modification was

mainly attributed to a decrease in the net negative charge on the modified enzyme resulting from the blocking of negatively charged carboxyl groups by glycine methyl ester.

When the number of carboxyl groups modified/mole pepsin was plotted against logarithm of the relative electrophoretic mobility, R_f , a linear relationship was obtained (Fig. 12). This indicates that the net charge of the pepsin molecule is directly proportional to the electrophoretic mobility. It has been shown that provided the molecular weight and molecular shape of a protein are known, the net charge of the protein can be calculated from its electrophoretic mobility (Shaw, 1969). The present result is in agreement with the above finding.

Presumably, the decrease in negative charge on pepsin was not only a result of the blocking of carboxyl groups by nucleophile but also of the fixation of positively charged carbodiimide residues as N-acylurea on other carboxyl groups of the pepsin molecule. Matyash et al. (1973) observed that pepsin treated with radioactive CMC alone contained 2-3 residues of labelled carbodiimide. In the present study, pepsin treated with 10 mM EDC in the absence of nucleophile had a R_f value of 0.89 (native pepsin was assumed to have a R_f of 1.0), showing that the contribution of carbodiimide to the decrease in net negative charge on pepsin was significant.

FIGURE 12. EFFECT OF CARBOXYL MODIFICATION OF PEPSIN ON RELATIVE ELECTROPHORETIC MOBILITY. NATIVE PEPSIN WAS ASSUMED TO HAVE A R_f VALUE OF 1.0.

Isoelectric Point of Native and Carboxyl Modified Pepsins

The isoelectric point of native pepsin, determined by electrophoresis of the protein on agarose gel at different pH's, was found to be below 0.5. After extensive carboxyl modification (11.2 carboxyl groups modified/mole pepsin), the isoelectric point was found to rise to about 0.7 (Fig. 13).

The present result is in accordance with that of other workers who observed that at pH 1.0, highly purified pepsin still migrated as an anion, indicating that the isoelectric point of porcine pepsin is below 1.0 (Tiselius et al., 1938; Herriott et al., 1940).

The isoelectric point of proteins determined by zone electrophoresis may be subject to errors such as absorption, capillary flow and electro-osmosis. More superior techniques would be moving boundary electrophoresis and isoelectrofocusing. However, while moving boundary electrophoresis requires expensive instruments and complicated experimental procedures, the pH range of commercially available ampholytes for isoelectrofocusing is between 3.5 and 10.0, well above the isoelectric point of pepsin. Agarose has been shown to exhibit little electro-osmosis and adsorption, and electrophoretic mobilities determined by agarose gel electrophoresis are found to be similar to those determined by moving boundary technique (Shaw, 1969).

FIGURE 13. DETERMINATION OF ISOELECTRIC POINTS OF NATIVE AND CARBOXYL MODIFIED PEPSINS.

●, NATIVE PEPSIN;

○, CARBOXYL MODIFIED PEPSIN (11,2 CARBOXYL GROUPS MODIFIED/MOLE ENZYME)

Thus, agarose gel electrophoresis was employed to provide information on changes in the isoelectric point of pepsin after chemical modification.

The increase in isoelectric point in the carboxyl modified pepsin further indicates that there was a decrease in the net negative charge in the enzyme as a result of modification.

Carboxyl Modification of Pepsin By Other Amino Acid Methyl Esters

Methyl esters of amino acids other than glycine were used as nucleophiles to modify pepsin. The extent of carboxyl modification, the activities and the relative electrophoretic mobility of the modified enzymes were measured and the results are presented in Table 5.

All nucleophiles were found to incorporate into pepsin, but the extent of modification was variable. The milk clotting activity of all modified enzymes was markedly reduced, particularly those modified with tyrosine and tryptophan methyl esters. At pH 2.0, the proteolytic activity against hemoglobin was not changed in pepsin modified with arginine and lysine methyl esters while pepsins treated with leucine, tyrosine and tryptophan methyl esters showed a drop in activity. The specific proteolytic activity at pH 3.5 was

Table 5. Carboxyl modification of pepsin by different amino acid methyl esters.^a

Methyl ester	No. COOH gp. modified/mole pepsin	Milk clotting activity (% control)	Proteolytic activity (% control)		R _f ^b
			pH 2.0	pH 3.5	
Arg	4.7	14	100	300	0.50
Lys	5.1	12	100	330	0.48
Leu	7.3	10	75	200	0.68
Tyr	8.0	7	50	100	0.68
Try	3.5	4	40	25	0.70
Gly	5.2	17	94	340	0.56

^a Pepsin (10 mg/ml) was modified with 33-fold excess of EDC and 174-fold excess of methyl ester at pH 5.5 for 90 minutes.

^b Native pepsin was assumed to have a R_f value of 1.0.

significantly increased in most modified enzymes, indicating a shift in pH profile. However, tyrosine methyl ester-treated pepsin did not show a change in proteolytic activity at pH 3.5 and the tryptophan derivative even showed a marked decrease in activity at this pH.

The difference in the response of pepsin to different types of amino acid methyl esters could be attributed to solubility. Incorporation of hydrophobic amino acids such as tyrosine and tryptophan into pepsin would increase the hydrophobicity and decrease the solubility of the enzyme, leading to an apparent drop in activity.

Alternatively, the discrepancy could be due to the covering of the hydrophobic binding site in pepsin by the hydrophobic methyl esters. Apart from the catalytic site, several investigators advocated a hydrophobic binding site in pepsin which plays an important role in pepsin activity (Tang, 1965; Jackson et al., 1966). The hydrophobic methyl esters would have a greater tendency to bind to this secondary substrate binding site, thus rendering it unavailable for further interaction with other substrates and resulting in a loss of activities.

The electrophoretic mobility of all modified enzymes was found to decrease when compared to the native enzyme. Arginine and lysine methyl esters, being cationic, decreased the electrophoretic mobility to a greater extent.

Caseinolytic Properties of Native and Carboxyl Modified Pepsins

1. Rate of casein hydrolysis

The rate of hydrolysis of 1% (w/v) whole casein by pepsin, determined by the release of non-protein nitrogen (NPN) soluble in 3% (w/v) TCA, was not significantly affected by carboxyl modification. As shown in Fig. 14 and Fig. 15, at both pH 5.3 and 6.5, there was an initial rapid rise in the release of nitrogen followed by a gradual increase. The extent of casein hydrolysis was considerably lower at pH 6.5 than at pH 5.3. At pH 5.3, the carboxyl modified enzyme hydrolysed casein at a rate slightly higher than that of the control (Fig. 14). At pH 6.5, however, the rate of casein hydrolysis was slightly decreased after modification (Fig. 15).

2. Electrophoretic patterns of hydrolysed casein

At time intervals when the release of NPN was determined, casein samples were also withdrawn simultaneously, solubilized in 6 M urea and subjected to agarose gel electrophoresis. The resulting patterns were essentially similar (Plate II). They all showed an initial conversion of κ -casein to para- κ -casein. This was followed by some breakdown of β -casein, the rate being faster with the modified pepsin. There was no perceptible breakdown of α_{s1} -casein.

FIGURE 14. RATE OF CASEIN HYDROLYSIS BY NATIVE AND CARBOXYL MODIFIED PEPSINS AT pH 5.3.

●, NATIVE PEPSIN;

○, CARBOXYL MODIFIED PEPSIN.

* NPN, NON-PROTEIN NITROGEN

FIGURE 15. RATE OF CASEIN HYDROLYSIS BY NATIVE AND CARBOXYL MODIFIED PEPSINS AT pH 6.5.

●, NATIVE PEPSIN;

○, CARBOXYL MODIFIED PEPSIN.

* NPN, NON-PROTEIN NITROGEN.

PLATE II. AGAROSE GEL ELECTROPHORETIC PATTERNS
OF WHOLE CASEIN HYDROLYSED BY NATIVE
AND CARBOXYL MODIFIED PEPSINS.

SAMPLE: 1, 1% (w/v) WHOLE CASEIN; 2, CASEIN
HYDROLYSED BY NATIVE PEPSIN (30 MIN.);
3, CASEIN HYDROLYSED BY NATIVE PEPSIN
(180 MIN.); 4, CASEIN HYDROLYSED BY
CARBOXYL MODIFIED PEPSIN (30 MIN.);
5, CASEIN HYDROLYSED BY CARBOXYL MODIFIED
PEPSIN (180 MIN.)

3. Electrophoretic patterns of milk curds

Reconstituted skimmilk was clotted with both native and carboxyl modified pepsins at pH 6.3. At time intervals after clotting, a sample of curd was withdrawn, solubilized in 6 M urea and applied to agarose plate for electrophoresis. The electrophoretic patterns, shown in Plate III, appeared to be identical for both native and modified enzymes. In both instances, there was a decline in the amount of κ -casein together with the appearance of a para- κ -casein band. This indicates that the nature of the clotting reaction was not changed by the modification.

The above results indicate that the caseinolytic properties of pepsin were not significantly affected by carboxyl modification. The decrease in milk clotting activity was therefore not due to the blocking of carboxyl group(s) essential for the hydrolysis of caseins in milk, since the rate of release of nitrogen from caseins was not decreased after modification.

In the present investigation, chymosin was found to have caseinolytic properties very similar to those of pepsin, since both the rate of casein hydrolysis and the electrophoretic patterns of hydrolysed caseins and curdled milk were similar. The result is consistent with the reports of other workers who compared the proteolysis of whole casein

PLATE III. AGAROSE GEL ELECTROPHORETIC PATTERNS OF MILK CURDS
PRODUCED BY NATIVE AND CARBOXYL MODIFIED PEPSINS.

SAMPLES 1,2,3,& 4: DISSOLVED CURDS FORMED WITH NATIVE PEPSIN;

SAMPLES 5,6,7,& 8: DISSOLVED CURDS FORMED WITH MODIFIED PEPSIN.

TIME AFTER CLOTTING: 1 & 5, 0 HR.; 2 & 6, 1/2 HR.;
3 & 7, 2 HR.; 4 & 8, 24 HR.

and individual casein fractions by porcine pepsin and calf rennet under different conditions. In most instances, pepsin was found to have approximately the same activity as rennet (Mickelsen and Fish, 1970; Hansen, 1970; Itoh and Thomasow, 1971; Green, 1972).

Coagulation of κ -Casein and α_{s1} -Casein by Native and Carboxyl Modified Pepsins

Since κ -casein has been known to be the protein component responsible for stabilization of the caseinate micelles (Waugh and von Hippel, 1956; Zittle, 1961; Zittle and Walter, 1963), an experiment was designed to test whether the diminished clotting activity of the carboxyl modified pepsin was due to a decrease in its ability to coagulate κ -casein.

The result shows that 0.2% (w/v) κ -casein at pH 5.3 and an ionic strength of 0.05 was coagulated by both native and modified pepsins. Omission of calcium in the reaction solution did not affect the results. The specific activity was markedly increased after modification (Table 6).

A 1:1 (w/w) mixture of κ -casein and α_{s1} -casein was also used as substrate since α_{s1} -casein is the calcium-sensitive component which is normally protected by κ -casein against coagulation (Zittle, 1961). In this case, carboxyl

Table 6. Effect of carboxyl modification on clotting activity of pepsin to κ -casein and κ - α_{s1} -casein mixture.

Enzyme	Substrate	* Specific activity	$\frac{\kappa + \alpha}{\kappa}$
Pepsin	0.2% κ -casein pH 5.3	1.2	-
COOH-modified pepsin	0.2% κ -casein pH 5.3	3.0	-
Pepsin	κ - α_{s1} -casein (1:1), pH 5.3	14.3	11.9
COOH-modified pepsin	κ - α_{s1} -casein (1:1), pH 5.3	1.5	0.5

* The casein clotting activity is defined as described under Method. Specific activity is expressed as clotting activity per mg protein.

modification greatly reduced the ability of pepsin to coagulate the casein mixture (Table 6).

The result also shows that native pepsin had a much higher specific activity against the casein mixture than against κ -casein, with an activity ratio $(\kappa + \alpha / \kappa)$ of about 12. In contrast, the carboxyl modified pepsin had an activity ratio of only 0.5, indicating that the enzyme had greater specificity towards κ -casein than κ - α_{s1} -casein mixture (Table 6).

When the specific activities against casein fractions were compared to those against skimmilk and hemoglobin, it can be seen that the rise in specific activity against κ -casein coincided with an increase in proteolytic activity at pH 3.5, with the exception of the most extensively modified enzyme which showed a drop of 70% in κ -casein clotting activity. The specific activity against κ - α_{s1} -casein mixture was found to decrease with a trend very similar to the drop in milk clotting activity (Table 7).

The results obtained from the above experiments suggest that the increase in specific activity against κ -casein was probably attributed to a shift in pH optimum of the enzyme similar to that observed when hemoglobin was used as substrate. Since the activity was measured at a pH (5.3) not far from the optimum pH (3.5) of the modified enzyme for proteolysis, the modified pepsin would hydrolyse κ -casein

Table 7. Comparision of activities of carboxyl modified pepsin to different substrates.

Enzyme	No. of COOH groups modified	Specific activity (% control)				
		κ -casein pH 5.3	κ - α_{s1} -casein pH 5.3	skimmilk pH 6.3	hemoglobin pH 2.0	hemoglobin pH 3.5
Pepsin	0	100	100	100	100	100
COOH modi- fied pepsin	2.8	320	20	27	88	210
"	5.2	250	12	17	94	340
"	8.6	200	10	13	105	330
"	11.2	30	5	9	90	340

at a rate faster than the native enzyme which has low proteolytic activity at higher pH's (see Fig. 9). The coagulation of isolated κ -casein by an enzyme has been demonstrated to be due to the aggregation of insoluble para- κ -casein split off from κ -casein (Cheeseman, 1962) and is therefore essentially proteolytic in nature. An increase in proteolytic activity against κ -casein would therefore increase the rate of κ -casein coagulation.

The dramatic drop in κ -casein clotting activity, when pepsin was extensively modified (11.2 moles of glycine methyl ester incorporated/mole pepsin), suggests that a carboxyl group(s) essential for κ -casein hydrolysis may be blocked. This group(s), however, was not essential for hydrolysing hemoglobin since the specific activity of this derivative against hemoglobin still remained high when compared to the native enzyme.

The high activity ratio ($\kappa+\alpha/\kappa$) observed for the native pepsin may suggest that interaction between κ -casein and α_{s1} -casein in the micelles could lead to changes that enhance the affinity of the native enzyme for the substrate. Activation of κ -casein hydrolysis by α_s -casein and β -casein was reported in chymosin (Kanamori et al., 1977) and was attributed to the association of κ -casein with other proteins.

The extensive loss in pepsin activity against κ -casein/ α_{s1} -casein mixture after carboxyl modification suggests that interaction between the two caseins on the micelles may retard the binding between the micelles and the modified enzyme. Changes in the charge distribution on the modified enzyme may interfere with substrate-enzyme interaction through electrostatic or ionic repulsion. This would affect the primary (enzymatic) phase of milk coagulation and slow down the secondary phase of micellar aggregation. The observed drop in milk clotting activity in the carboxyl modified pepsin could therefore be attributed to a change in the ionic characteristic of the enzyme.

Specificity of Carboxyl Modification

To investigate whether the carboxyl modification by the carbodiimide reaction is specific for pepsin, pepsinogen and chymosin were modified under similar conditions (33-fold excess of EDC, 716-fold excess of glycine methyl ester, pH 5.5, 90 minutes incubation).

The result, presented in Table 8, indicates that the carbodiimide reaction was not specific for pepsin. Both pepsinogen and chymosin were modified to about the same extent as pepsin with a significant decrease in milk clotting activity. The decrease in proteolytic activity

Table 8. Effect of carboxyl modification on activities of pepsin, pepsinogen and chymosin.*

Enzyme	No. of COOH gp. modified	<u>Specific activity (% control)</u>		
		Milk clotting pH 6.3	<u>Proteolytic</u> pH 2.0 pH 3.5	
Pepsin	11.2	9	90	340
Pepsinogen	12.1	30	70	450
Chymosin	13.0	15	50	50

* The enzymes (10 mg/ml) were modified with 33-fold excess of EDC and 706-fold excess of glycine methyl ester at pH 5.5 for 90 minutes.

of the modified zymogen and chymosin at pH 2.0 was not as extensive as the drop in milk clotting activity. Like the modified pepsin, the proteolytic activity of the modified pepsinogen at pH 3.5 was significantly increased, suggesting a shift in pH profile. In contrast, modified chymosin only had 50% proteolytic activity at pH 3.5 when compared to the native enzyme. Native chymosin has a proteolytic pH optimum near 3.5 (Berridge, 1945), a shift in pH optimum, if occurred in the modified chymosin, would be detected at pH lower or higher than 3.5.

Both carboxyl modified pepsinogen and chymosin exhibited a marked retardation in electrophoretic mobility in agarose gels with R_f values of 0.70 and 0.63 respectively (compared to the native proteins which were assumed to have a R_f value of 1.0). This indicates a decrease in net negative charge on the modified protein molecules.

The results show that the carbodiimide reaction was not specific for pepsin. Both the pepsin precursor and other acid proteases such as chymosin, when modified, showed similar responses. In contrast to the present finding, modification with CMC and a colored amine was found to be specific to pepsin (Matyash et al., 1973). No amine was incorporated into pepsinogen and an acid protease from Aspergillus awamori. The zymogen and fungal protease retained 100% proteolytic activity. This suggests that

CMC and the colored amine are only effective in modifying the carboxyl groups in pepsin. On the other hand, EDC and glycine methyl ester are able to modify the carboxyl groups in pepsin and related enzymes, showing that they are less selective than the CMC-colored amine system.

Effect of Carboxyl Modification on K_m and k_{cat} of Pepsin

For kinetic studies, the synthetic dipeptide APDT was used as substrate. The Michaelis constant (K_m) and molecular activity coefficient (k_{cat}) were determined at pH 2.0 and 4.5. APDT was chosen because it is among the most sensitive substrates for pepsin (Fruton, 1970) and the kinetic parameters of pepsin on this substrate have been determined (Jackson *et al.*, 1965).

Fig. 16 and Fig. 17 depict the Lineweaver-Burk plots for native and carboxyl modified pepsins at pH 2.0 and 4.5 respectively. The K_m values determined from the intercepts are presented in Table 9. The k_{cat} values, calculated from the Michaelis-Menten equation, are also shown in Table 9.

The K_m and k_{cat} values determined for native pepsin were found to compare favorably with the published data (Jackson *et al.*, 1965). The result shows that after carboxyl modification, K_m was markedly increased at both pH

FIGURE 16. LINEWEAVER-BURK PLOTS OF THE HYDROLYSIS OF N-ACETYL-L-PHENYLALANYL-DIIODO-L-TYROSINE BY NATIVE ($\blacktriangle$) AND CARBOXYL MODIFIED ($\triangle$) PEPSINS AT pH 2.0.

FIGURE 17. LINEWEAVER-BURK PLOTS OF THE HYDROLYSIS OF N-ACETYL-L-PHENYLALANYL-DIIODO-L-TYROSINE BY NATIVE ($\blacktriangle$) AND CARBOXYL MODIFIED ($\triangle$) PEPSINS AT pH 4.5.

Table 9. Kinetics of the hydrolysis of N-acetyl-L-phenylalanyl-L-diiiodotyrosine by native and carboxyl modified pepsins.

Enzyme	Concn. μM	pH	K_m $\text{M} \times 10^6$	k_{cat} min.^{-1}
Pepsin	0.51	2.0	0.78	12.0
COOH modified pepsin	0.76	2.0	1.33	13.8
Pepsin	0.76	4.5	12.0	4.1
COOH modified pepsin	1.52	4.5	37.0	4.2

2.0 and 4.5, while k_{cat} was not markedly altered.

The increase in K_m suggests that carboxyl modification of pepsin decreased the affinity of the enzyme for the dipeptide substrate, i.e., the enzyme-substrate binding step was hindered. On the other hand, the catalysis of the enzyme-substrate complex was not affected, as the k_{cat} values were not changed markedly after carboxyl modification. The partial loss in peptidase activity in the modified pepsin could be attributed to a blocking of the active site on the enzyme.

A change in kinetic parameters was also reported in arginine modified pepsin which has 45% of the proteolytic activity of the native pepsin (Kitson and Knowles, 1971). In this case, however, K_m was not changed while k_{cat} was significantly lowered, indicating that the inactivation took place not by the blocking of the enzyme's active site, but by an effect on the catalytic process subsequent to enzyme-substrate binding.

Effect of Synthetic Dipeptides on Carboxyl Modification

Carboxyl modification of pepsin was carried out in the presence of some synthetic dipeptides. These include a sensitive substrate (APDT), a poor substrate (N-carbo-benzoxy-L-glutamyl-L-tyrosine, Z-glu-tyr) and a non-substrate (N-acetyl-D-phenylalanyl-L-tyrosine, APT). Modifications were carried out under the same conditions (33-fold excess EDC, 174-fold excess glycine methyl ester, pH 5.5 and 90 minutes). The extent of modification, milk clotting activity and electrophoretic mobility of pepsins after treatments were determined (Table 10).

Result shows that in the presence of APDT, a sensitive pepsin substrate, the extent of carboxyl modification was significantly decreased when compared to pepsin modified in the absence of substrate. The milk clotting activity and electrophoretic mobility, markedly decreased by carboxyl modification, were only moderately lowered when modification took place in the presence of APDT.

In the presence of a poor substrate and a non-substrate, pepsin was also modified, but to a lesser extent than the fully modified control. The milk clotting activity and R_f value were increased relative to the fully modified pepsin. However, the changes were not as marked as those that occurred in the presence of APDT.

Table 10. Effect of dipeptides on carboxyl modification of pepsin.

Addition	Concn. mM	No. of COOH gp. modified	Milk clotting activity (% control)	R _f
1. None	-	0	100	1.00
2. EDC + gly methyl ester	10 50	5.2	17	0.56
3. (2) + APDT ^a	0.3	2.3	50	0.90
4. (2) + Z-glu-tyr ^b	2	3.6	35	0.77
5. (2) + APT ^c	2	4.8	22	0.65

^a N-acetyl-L-phenylalanyl-diiodo-L-tyrosine.

^b N-carbobenzoxyl-L-glutamyl-L-tyrosine.

^c N-acetyl-D-phenylalanyl-L-tyrosine.

The present result indicates that the effect of carboxyl modification on pepsin properties were less prominent in the presence of a dipeptide substrate. This could be due to one of the following. Firstly, the binding of the dipeptide substrate to the enzyme may protect the carboxyl groups, mainly those in the vicinity of the pepsin active centre from modification by carbodiimide and nucleophile. Secondly, substrate-enzyme binding may lead to conformational changes in the pepsin molecule which indirectly affect subsequent modification. Finally, the substrate may interact with carbodiimide and nucleophile and less reagents would be available for modifying pepsin.

The result shows that even non-substrate can hinder carboxyl modification, though to a lesser extent than the two dipeptide substrates. This suggests that dipeptides can react with the reagents. APDT caused more pronounced changes than Z-glu-tyr probably because it is a better substrate and can bind to pepsin more efficiently. Hence, substrate-enzyme binding would be the major factor in causing such moderation in carboxyl modification.

Response of Native and Carboxyl Modified Pepsins to Inhibitors

The reactions of the native and carboxyl modified pepsins with inhibitors were studied. Two pepsin inhibitors, 1,2-epoxy-3-(p-nitrophenoxy) propane (EPNP) and bromophenacyl bromide were used. EPNP is a substrate-like epoxide inactivator modifying Asp-76 in pepsin with almost complete loss of proteolytic activity (Chen and Tang, 1972), while bromophenacyl bromide reacts with a carboxyl group in pepsin not directly involved in catalysis (Erlanger *et al.*, 1965; Clement, 1973).

Fig. 18 shows the inactivation of native and carboxyl modified pepsins by EPNP. Native pepsin was inactivated more rapidly and the treated enzyme retained only 10% of proteolytic activity after 120 hours of incubation. The modified enzyme reacted less promptly with EPNP, and retained 30% of its activity at the end of incubation.

Table 11 shows the reaction of pepsin with EPNP and bromophenacyl bromide. Bromophenacyl bromide produced a loss of 70 % proteolytic activity in the native pepsin, but only 35% in the modified enzyme.

The result indicates that carboxyl modified pepsin was still reactive to site-specific inactivators, confirming that the carboxyl groups blocked were not the active-site residues of pepsin. However, the modified pepsin was less reactive to the inactivators than the native enzyme. This suggests

FIGURE 18. INACTIVATION OF NATIVE AND CARBOXYL
MODIFIED PEPSINS BY EPNP.

●, NATIVE PEPSIN;

○, CARBOXYL MODIFIED PEPSIN.

Table 11. Response of native and carboxyl modified pepsins to inhibitors.

Enzyme	Inhibitor	Concn.	Proteolytic activity (% remaining)
Pepsin	EPNP ^a	1 mg/mg protein	10
COOH modified pepsin	EPNP	1 mg/mg protein	27
Pepsin	BPB ^b	200 μ M	30
COOH modified pepsin	BPB	200 μ M	65

^a 1,2-epoxy-3-(p-nitrophenoxy) propane.

^b bromophenacyl bromide.

that the carboxyl groups reactive to the inhibitors were protected in the modified enzyme, probably as a result of conformational change or steric hindrance.

Stability of Native and Carboxyl Modified Pepsins Near Neutral pH

The stability of pepsin near neutral pH was studied. In the first series of experiments, pepsin was incubated in dilute buffer (0.05 M phosphate buffer) at pH 6.5 and 30° C. Both milk clotting and proteolytic activities of pepsin were determined at selected time intervals. The percentage changes in milk clotting and proteolytic activities were found to be similar. Subsequently, only milk clotting activity was determined.

Fig. 19 shows the stability curves of native and carboxyl modified crystalline pepsins in buffer. The milk clotting activity of native pepsin dropped rapidly, following a first order kinetic with a half-life ($t_{1/2}$) for inactivation of about 15 minutes. Less than 10% activity was retained after one hour of incubation. With mild modification (incorporation of 2.8 moles of nucleophile/mole pepsin), the stability was markedly improved, and the enzyme retained 20% of milk clotting activity after 4 hours. When pepsin was more extensively modified (incorporation of 5.2 moles of nucleophile), the stability was improved further.

FIGURE 19. STABILITY OF CRYSTALLINE NATIVE AND CARBOXYL MODIFIED PEPSINS IN 0.05 M PHOSPHATE BUFFER, PH 6.5. ▲, NATIVE PEPSIN; △, PEPSIN MODIFIED WITH 87-FOLD EXCESS OF NUCLEOPHILE; ■, PEPSIN MODIFIED WITH 174-FOLD EXCESS OF NUCLEOPHILE.

The curve started to level off after 2 hours and the loss in activity was only about 50% after 4 hours of incubation.

The stability of crude pepsin in dilute buffer was also studied. As shown in Fig. 20, the crude pepsin preparation had a low stability at pH 6.5. The time-dependent decrease in milk clotting activity also followed a first order kinetic. The half-life of inactivation was calculated to be 10 minutes. The stability was again found to increase markedly after carboxyl modification. The degree of improvement was also dependent on the extent of modification. The less extensively modified enzyme retained 25% activity after 2 hours incubation while the more extensively modified pepsin retained about 50% activity.

In the second series of experiments, pepsins were incubated in milk ultrafiltrate at 30°C with progressive adjustment of pH according to that found in cheese-making. The result for crystalline pepsins is presented in Fig. 21 and that for crude pepsins is shown in Fig. 22. In both cases, the stability of the native enzyme was much higher in milk ultrafiltrate than in dilute buffer. The milk clotting activity decreased rapidly in the first hour but started to level off after 2 hours. For both crystalline and crude pepsins, about 20% activity was found to remain after incubation.

FIGURE 20. STABILITY OF CRUDE NATIVE AND CARBOXYL MODIFIED PEPSINS IN 0.05 M PHOSPHATE BUFFER, pH 6.5.

▲, NATIVE PEPSIN; △, PEPSIN MODIFIED WITH 87-FOLD EXCESS OF NUCLEOPHILE; ■, PEPSIN MODIFIED WITH 174-FOLD EXCESS OF NUCLEOPHILE.

FIGURE 21. STABILITY OF CRYSTALLINE NATIVE AND CARBOXYL MODIFIED PEPSINS IN MILK ULTRAFILTRATE UNDER SIMULATED CHEESE-MAKING CONDITIONS.

●, NATIVE PEPSIN;

○, CARBOXYL MODIFIED PEPSIN.

FIGURE 22. STABILITY OF CRUDE NATIVE AND CARBOXYL MODIFIED PEPSINS IN MILK ULTRAFILTRATE UNDER SIMULATED CHEESE-MAKING CONDITIONS.

●, NATIVE PEPSIN;

○, CARBOXYL MODIFIED PEPSIN.

After carboxyl modification, the stability of both crystalline or crude pepsin was markedly improved. The rate of decline in activity was more gradual when compared to the control. For both enzymes, the decrease in activity was about 40%.

The results show that the stability of pepsin near neutral pH was much improved by carboxyl modification. Pepsin was shown to be unstable at pH 6.0 (Herriott, 1955) and was rapidly denatured at pH 7.0 at 30° C (O'Leary and Fox, 1974). It was suggested that an electrostatic expansion of the negatively charged polypeptide chains may lead to denaturation of pepsin at pH values higher than 6 (Lowenstein, 1974). The decrease in net negative charge on the carboxyl modified pepsin molecule may lower the extent of electrostatic expansion and subsequent denaturation.

An improvement of enzyme stability at pH 6.3-6.5 was also reported in porcine pepsin covalently bound to a soluble polyanionic carrier, ethylene maleic anhydride (Lowenstein, 1974). By incubating the enzyme in 0.05 M phosphate buffer of pH's 6.3 and 6.5 at 30° C, it was observed that the stability against the irreversible denaturation was 3-5 times better after modification. The enhanced stability was attributed to different microenvironmental states of the enzymes.

The higher stability of native pepsin in milk ultrafiltrate than in dilute buffer was probably due to the protection of the enzyme by other milk solids against denaturation. The estimation of the amount of inactivation of pepsin in milk ultrafiltrate under simulated cheese-making conditions would therefore give a more valid assessment of the suitability of the modified enzyme as a milk coagulant for cheese manufacture. However, there were differences between the simulated test and conditions that actually exist during cheese-making.

Firstly, acid was added at intervals in the simulation while in the actual process, acid was produced continuously, although this probably has little influence on the result. Secondly, the temperature was kept constant at 30°C in the simulation but is raised for scalding in cheese-making, although the rise in temperature would be expected to reduce the amount of active enzyme to a greater extent. Thirdly, the environment of the enzyme in the simulation was different from that in cheese-making. The concentrations of enzyme were lower and other proteins were present in higher concentrations in the curd, which might increase the stability of the enzyme. Finally, the results may not represent the amount of active enzyme retained in the curd, since the distribution of enzyme between whey and curd was not known.

However, in spite of these provisions, the present results indicate that native pepsin was extensively inactivated under cheese-making conditions while the carboxyl modified pepsin was a much more stable enzyme. Consistent with the present finding, Green (1972) reported that crude porcine pepsin (1:3,000) was almost completely denatured in milk dialysate under simulated cheese-making conditions while rennet was found to be fairly stable, retaining about 60% of proteolytic activity after treatment. In another report, Holmes and Ernstrom (1973) were unable to recover any active enzyme from Cheddar curd made with pig pepsin whereas 5% of the added calf rennet was recovered.

It has been observed that cheese made with porcine pepsin has a harder body, developed flavor slowly and required an aging period longer than was necessary with calf rennet (Maragoudakis et al., 1961; Melachouris and Tuckey, 1964; Emmons et al., 1971; Green, 1972). It was suggested that in cheese made with calf rennet, the active enzyme retained in the curd would aid the starter enzymes in the ripening of cheese, while in pepsin cheese, the proteolytic breakdown essential for ripening would be almost entirely dependent on starter activity (Green, 1972; Lawrence et al., 1972). Hence, the long aging period required in pepsin cheese has been attributed to a slow rate of proteolysis as a result of extensive inactivation of the enzyme during

cheese-making (Thomasow, 1971; Green and Foster, 1974).

The carboxyl modified pepsin might be a better milk coagulant than the native enzyme in cheese manufacture. Since the stability at pH above 6 was significantly improved, a greater amount of active enzyme would be retained in the curd to aid in the ripening of cheese, with a shortening in the aging period and a reduction in production cost. A more valid and complete comparison between native and carboxyl modified pepsins in their performance in cheese manufacture would await further investigations with small scale cheese-making trials.

Thermal Stability of Native and Carboxyl Modified Pepsins

The stability of pepsin at elevated temperatures was studied. Both milk clotting and proteolytic activities were measured.

Fig. 23 and Fig. 24 show the thermal profiles (milk clotting) of crystalline and crude pepsins respectively. For crystalline pepsin, both native and modified enzymes showed a maximum milk clotting activity at about 40° C, and a rapid drop from 45 to 50° C. The percentage maximum activity was higher in the native pepsin than in the modified enzyme at all temperatures except at 50° C (Fig. 23).

FIGURE 23. THERMAL PROFILES (MILK CLOTTING) OF CRYSTALLINE NATIVE AND CARBOXYL MODIFIED PEPSINS.

●, NATIVE PEPSIN;

○, CARBOXYL MODIFIED PEPSIN.

FIGURE 24. THERMAL PROFILES (MILK CLOTTING) OF CRUDE NATIVE AND CARBOXYL MODIFIED PEPSINS.

●, NATIVE PEPSIN;

○, CARBOXYL MODIFIED PEPSIN.

The thermal profiles for crude pepsin were similar to those of the crystalline enzymes. The native crude pepsin had a maximum activity at 40°C . After modification, the maximum was shifted to 45°C . The percentage maximum activity was considerably higher in the native pepsin than in the modified enzyme at temperatures between $25-40^{\circ}\text{C}$ but lower at $45-50^{\circ}\text{C}$ (Fig. 24).

The Q_{10} values (milk clotting) for both crystalline and crude pepsins are presented in Table 12. The results indicate that the Q_{10} values increased after carboxyl modification. The increases were particularly marked at the higher temperature range ($40-50^{\circ}\text{C}$). This shows that modified pepsin had higher heat stability than the native enzyme with regard to milk clotting activity.

The thermal profiles for proteolysis are shown in Fig. 25 and Fig. 26. For crystalline pepsins, the percentage maximum activity was higher in carboxyl modified enzyme at lower temperatures ($30-50^{\circ}\text{C}$). At higher temperatures ($50-70^{\circ}\text{C}$), however, the proteolytic activity of the native pepsin was greater than that of carboxyl modified pepsin. Both enzymes showed a rapid decrease in activity from $60-70^{\circ}\text{C}$ (Fig. 25). For crude pepsins, the thermal profiles of the native and modified enzymes were almost identical. Both enzymes showed a gradual increase in proteolytic activity with increasing temperature up to 60°C . From 60°C to

Table 12. Effect of carboxyl modification of pepsin on Q_{10} values (milk clotting).

Enzyme	Temperature ($^{\circ}\text{C}$)	Q_{10}^*
Pepsin (crystalline)	30-40	1.6
	40-50	0.4
COOH modified pepsin (crystalline)	30-40	1.9
	40-50	0.6
Pepsin (1:10,000)	30-40	1.3
	40-50	0.4
COOH modified pepsin (1:10,000)	30-40	1.6
	40-50	0.7

* Q_{10} values represent the ratio of milk clotting activity at $(T+10)^{\circ}$ to that at T° .

FIGURE 25. THERMAL PROFILES (PROTEOLYTIC) OF CRYSTALLINE NATIVE AND CARBOXYL MODIFIED PEPSINS.

●, NATIVE PEPSIN;

○, CARBOXYL MODIFIED PEPSIN.

FIGURE 26. THERMAL PROFILES (PROTEOLYTIC) OF CRUDE
NATIVE AND CARBOXYL MODIFIED PEPSINS.

●, NATIVE PEPSIN;

○, CARBOXYL MODIFIED PEPSIN.

70°C, the activity decreased sharply to less than 20% of the maximum level (Fig. 26).

The Q_{10} values for proteolysis are presented in Table 13. For both crystalline and crude pepsins, the native enzymes had higher Q_{10} values than the modified enzymes at all temperature ranges. The difference was not marked except at 60-70°C when the Q_{10} value of native crystalline pepsin was three times higher than that of the modified enzyme. The result shows that carboxyl modification caused a slight drop in thermal stability of pepsin when the activity was measured against hemoglobin.

The rapid decline in milk clotting and proteolytic activities of pepsin at high temperatures was due to denaturation. At pH 6.3 at which milk clotting activity was measured, denaturation occurred at temperatures above 45°C. At pH 2.0 at which proteolytic activity was measured, denaturation did not occur until the temperature exceeded 60°C. This indicates that pepsin was more stable against heat at lower pH's. Similar phenomena were reported by O'Keeffe et al. (1977) who showed that denaturation of pepsin was critically dependent on pH and temperature within very narrow limits.

The improvement in heat stability of carboxyl modified pepsin at milk clotting pH may be of some practical

Table 13. Effect of carboxyl modification of pepsin on Q_{10} values (proteolytic).

Enzyme	Temperature ($^{\circ}\text{C}$)	Q_{10}^*
Pepsin (crystalline)	30-40	1.43
	40-50	1.10
	50-60	1.21
	60-70	0.54
COOH modified pepsin (crystalline)	30-40	1.27
	40-50	1.07
	50-60	0.90
	60-70	0.17
Pepsin (1:10,000)	30-40	1.37
	40-50	1.04
	50-60	1.11
	60-70	0.16
COOH modified pepsin (1:10,000)	30-40	1.31
	40-50	1.00
	50-60	1.09
	60-70	0.15

* Q_{10} values represent the proteolytic activity at $(T+10)^{\circ}$ to that at T° .

importance. In the making of Cheddar cheese, the curd is normally heated to temperatures near 40°C during cooking, while the pH remains above 6.0. An increase in enzyme stability under these conditions would ensure that a higher proportion of active enzyme to be retained in the curd and be available for aiding the ripening of cheese.

Effect of Carboxyl Modification on Curd Tension and Rate of Syneresis

The firmness of curds from milk clotted by native and carboxyl modified pepsins was measured by the method of Hehir (1968). The result, shown in Table 14 indicates that for both crystalline and crude pepsins, there was no significant difference between the firmness of curds from native and modified enzymes. For crystalline pepsins, the native enzyme produced a slightly firmer curd than the modified enzyme, while the reverse was demonstrated for the crude pepsins. According to the comparison of curd tension measurements with subjective assessment of curd firmness (Hehir, 1968), a curd tension higher than 75 units (cg) corresponds to a firm curd. Hence, both crystalline and crude pepsins produced a firm curd and the curd tension was comparable to that of chymosin which gave a firmer curd (Table 14).

Table 14. Tension of curds produced by pepsin, carboxyl modified pepsin and chymosin.

Enzyme	Clotting time (min.)	Curd tension (cg)
Pepsin (crystalline)	3.5	125 $\pm$ 5*
COOH modified pepsin (crystalline)	3.7	118 $\pm$ 4
Pepsin (1:10,000)	4.5	98 $\pm$ 4
COOH modified pepsin (1:10,000)	4.5	107 $\pm$ 6
Chymosin	3.5	135 $\pm$ 6

* Curd tension values are presented as averages of five determinations $\pm$ S.E.

The effect of carboxyl modification of pepsin on the rate of syneresis was studied. The results are presented in Fig. 27 and Fig. 28. For crystalline pepsins, the rate of syneresis was not markedly affected by modification. The native and modified enzymes produced almost identical curves (Fig. 27). The percentage syneresis increased rapidly in the first two hours and then levelled off. The modified pepsin produced syneresis at a rate faster than the control, but the final percentage syneresis was slightly lower than that of the native enzyme. Chymosin was found to yield syneresis at a rate very similar to that of pepsin (Fig. 27).

For crude pepsins, the percentage syneresis was considerably lower than that of the crystalline pepsins. Syneresis developed at a slower rate, and the curves did not level off until after three hours. There was no significant change in the syneresis rate after carboxyl modification. The modified enzyme produced syneresis at a rate faster than the control, while the final percentage syneresis was again slightly lower than that of the native enzyme (Fig. 28).

The above results show that in the initial stages of cheese-making, carboxyl modification of pepsin did not affect the quality of the curd such as curd tension and the rate of exudation of whey.

FIGURE 27. SYNERESIS OF CURD BY CRYSTALLINE PEPSIN, CARBOXYL MODIFIED CRYSTALLINE PEPSIN AND CHYMOSIN.

- ▲, NATIVE PEPSIN;
- △, CARBOXYL MODIFIED PEPSIN;
- , CHYMOSIN.

FIGURE 28. SYNERESIS OF CURD BY CRUDE NATIVE AND CARBOXYL MODIFIED PEPSINS.

▲, NATIVE PEPSIN;

△, CARBOXYL MODIFIED PEPSIN.

GENERAL DISCUSSION

In the present investigation, selective modification of the carboxyl groups in porcine pepsin was carried out at pH 5.5 using a water-soluble carbodiimide, EDC and an amino acid methyl ester as nucleophile. The number of nucleophile molecules incorporated per molecule of pepsin ranged from 2.8 to 11.2. Profound changes in the activities, specificity and physicochemical properties were observed in the carboxyl modified pepsin.

There was a significant decrease in milk clotting activity while the proteolytic activity against hemoglobin was not affected. The clotting activity against κ -casein was increased by two to three-fold while the rate of coagulation of κ - α_{s1} -casein mixture was decreased markedly to 10-20% of the control. There was a shift in proteolytic pH profile with the pH optimum increased from 2.0 to about 3.5. The relative electrophoretic mobility was decreased and the isoelectric point was slightly increased. There was a drop in peptidase activity and the K_m was increased while K_{cat} was not changed. Finally, the pH stability of the enzyme was significantly increased.

Several lines of evidence suggest that the drop in milk clotting activity of the modified pepsin was not

directly related to the modification of specific carboxyl group(s) involved in the enzymatic clotting of milk.

Firstly, the rate of casein hydrolysis was not affected by carboxyl modification, and both native and modified pepsin showed similar caseinolytic properties. This indicates that the chemical procedure did not modify the carboxyl group(s) involved in the enzymatic breakdown of casein which is the first step in the coagulation of milk mediated by an enzyme.

Secondly, pepsin treated with EDC alone showed a slight drop in milk clotting activity although the carboxyl residues were not modified. The treated enzyme had a slightly lower electrophoretic mobility than the control, suggesting a decrease in net negative charge on the enzyme molecule. The incorporation of the positively charged carbodiimide on the pepsin might lead to a change in the charge distribution of the enzyme which could affect the milk clotting activity.

Finally, the carboxyl modification was not specific to pepsin. It caused similar drop in milk clotting activity of pepsinogen and chymosin. If the decrease in milk clotting activity of the modified pepsin was due to the blocking of specific carboxyl group(s) responsible for the enzymatic coagulation of milk, modification of other enzymes may not cause similar changes. This was illustrated by

Matyash et al. (1973) who found that modification of pepsin carboxyl groups by CMC and a colored amine resulted in a drop in proteolytic activity, while similar treatment on pepsinogen and another acid protease did not cause changes in activity.

In order to explain the decrease in milk clotting activity of the carboxyl modified pepsin, one has to understand the mechanism of milk clotting process. Curdling of milk by clotting enzymes is a complex phenomenon. The first step or primary phase involves a highly specific action of enzyme on κ -casein to destroy the micelle-stabilizing power of the protein. κ -Casein, a glycoprotein, is split specifically at the Phe-Met bond. This results in the release of an insoluble para- κ -casein and a soluble glycomacropeptide.

The sequence of events following the enzymatic action on κ -casein to destabilize the micelles is poorly understood. This is partly due to an incomplete understanding of the structure of the caseinate micelle. Several models have been proposed (Waugh and Noble, 1965; Garnier and Ribadeau-Dumas, 1970; Parry and Carrell, 1969; Slattey and Evard, 1973) but no one is absolutely satisfactory.

However, from the available experimental data, some facts about the secondary phase are now known. Apparently,

coagulation requires the conversion of a minimum amount of κ -casein to para- κ -casein (Green and Marshall, 1977). The release of the highly negatively charged glycomacropptides from micelles decreases the negative charge on the micelles (Green and Crutchfield, 1971; Pearce, 1976). This results in the reduction of electrostatic repulsion between micelles and promotes aggregation (Green and Crutchfield, 1971). Para- κ -casein, present on the micellar surface, increases the hydrophobicity of the particles, again promoting aggregation through hydrophobic interactions (Payens, 1966). It has been suggested that coagulation results from specific interaction between micelles through enzymatically-modified areas on their surfaces (Waugh, 1971; Knoop and Peters, 1975; Green and Marshall, 1977).

From the above observations, one may speculate that the decrease in the rate of milk coagulation by carboxyl modified pepsin is attributed to a slow down in the release of para- κ -casein resulting from an interference with the enzyme-micelle interaction. Since a defined amount of para- κ -casein is required to initiate coagulation (Green and Marshall, 1977), a drop in the rate of para- κ -casein release would directly affect the aggregation of the micelles.

Alternatively, the decrease in milk clotting activity could be related to the secondary stage of coagulation, i.e., interactions of the enzymatically modified micelles and the subsequent aggregation to form curd. The carboxyl modified pepsin may not be able to produce the suitable types of micelles for micelle-micelle interaction, which is viewed as a highly specific reaction (Knoop and Peters, 1975; Green and Marshall, 1977).

Results from casein coagulation experiments indicate that κ -casein was clotted by the modified pepsin at a rate faster than the native enzyme, while the κ - α_{s1} -casein mixture was much more resistant to clotting by the modified enzyme. The clotting activity against the casein mixture was decreased to the same extent as the drop in milk clotting activity after carboxyl modification (see Table 7). The data suggest that κ - α_{s1} -casein interaction in the micelles may cause alterations that increase the affinity of native pepsin for its substrate, but hinder the binding between the micelles and the modified enzyme. This is consistent with the view that the decrease in milk clotting activity is related to an interference with the enzyme-micelle interaction.

The present results suggest that the clotting of milk is a process in which charge may play an important role. At least part of the forces involved in micelle-

enzyme interaction in the primary phase would be electrostatic in nature, and changes in the charge distribution on the enzyme molecule could cause profound alteration in the vital substrate-enzyme binding process. This is consistent with the work of Green and Marshall (1977) who observed an increase in the affinity of calf rennet for the micelles and an acceleration in the aggregation of caseinate micelles by the addition of cationic materials, indicating that charge plays an important role in the clotting of milk.

In contrast to milk clotting, hydrolysis of proteins such as denatured hemoglobin by pepsin did not seem to be significantly influenced by charge effect. Although the pH profile was shifted, the specific proteolytic activity at the optimum pH was relatively unchanged after carboxyl modification. The result suggests that coagulation of milk by pepsin, although involving a vital proteolytic step, is different from general proteolysis, probably in the mode of binding between enzyme and different substrates.

Apart from the decrease in milk clotting activity, changes in some physicochemical properties in the carboxyl modified pepsin are also attributed to an alteration in the net charge carried by the enzyme. The shift in pH activity curve when hemoglobin was used as substrate, the decrease in relative electrophoretic mobility on agarose

gel and the slight increase in isoelectric point could all be related to a change in the net ionic charge on the modified enzyme.

Modification of individual charged groups of proteins usually affect the net ionic charge in a way which may be disruptive to their characteristic properties. Decreased solubility and changes in conformation or in the state of aggregation frequently result from such modifications (Means and Feeney, 1971). These changes were not observed in carboxyl modified pepsin, except when tyrosine and tryptophan methyl esters were incorporated into the enzyme, which resulted in a significant decrease in solubility. This, however, was probably not attributed to the charge effect but to an increase in the hydrophobicity of the enzyme upon the incorporation of hydrophobic groups.

Kinetic study on the modified pepsin suggests that the drop in peptidase activity was possibly due to a decrease in the affinity of the modified enzyme to the dipeptide substrate, as indicated by the increase in K_m . This may be due to either a change in the conformation or the charge distribution on the enzyme, both of which could affect the binding step between the enzyme and the substrate. The fact that K_{cat} remained unchanged indicates that the decrease in peptidase activity was not due to a change in the catalytic step.

The kinetic data cannot be applied directly to explain the drop in milk clotting activity in the modified pepsin since the substrates used were different. If the K_m for milk coagulation can be calculated and found to increase after carboxyl modification, one can conclude that the drop in milk clotting activity was due to an interference with the binding between the enzyme and the micelles.

Results from the present investigation show that carboxyl modification of pepsin greatly improves the stability of the enzyme at pH around 6.5. This may have important practical implication related to the use of this enzyme as a milk coagulant in cheese-making.

The traditional coagulant used for cheese-making since pre-historic time is rennet extract from the abomasa of 10 to 30-day-old milk-fed calves. With a rapid rise in world-wide cheese consumption, coupled with a decrease in the practice to slaughter newly born calves, there has been a chronic world shortage of rennet during the last two decades. This has stimulated interest in finding suitable substitutes for rennet to use as coagulants in the making of cheese.

The basic function of a clotting enzyme is the conversion of liquid milk to a gel. This process can be

catalysed by most proteases. Apart from clotting milk, a suitable coagulant has to survive through cheese-making, and the residual enzyme incorporated in the curd should contribute to proteolysis of the cheese during ripening (Lawrence et al., 1972; Green and Foster, 1974). However, many proteases are too proteolytic at the customary pH values of milk and cheese, hence reducing the yield of cheese and retention of fat by the curd (Veringa, 1961; Ritter, 1970), and generating bitter peptides and poor body (Green and Foster, 1974). Thus, the most useful milk coagulants have a high clotting to proteolytic enzyme ratio (Ernstrom, 1974).

Proteolytic enzymes from animals, higher plants and micro-organisms have been studied for their suitability to replace calf rennet in making cheese, but only a few were successful commercially. These are swine pepsin, used as 50:50 rennet-pepsin mixtures, and fungal rennets from Endothia parasitica (Sardinas, 1968), Mucor pusillus Lindt (Oka et al., 1973; Arima et al., 1976) and Mucor meihei (Sternberg, 1972).

Recent literature indicates that good quality cheese can sometimes be produced using fungal rennets. In some cases, however, cheeses manufactured with microbial rennets were of a slightly lower quality than calf rennet cheeses (Martens and Naudts, 1976).

The use of pepsin, particularly porcine and bovine pepsins in cheese-making was considered a long time ago. In recent years, the use of calf rennet mixed with swine pepsin became wide-spread, and has been found to produce good quality cheeses of various types including Cheddar (Phelan, 1973), Pharmigiano-Reggiano (Corradini et al., 1976), Grana, Mozzarella and Taleggiano cheeses (Bottazzi et al., 1976). Recently, pure bovine pepsin was used on an industrial scale in the production of Cheddar (Emmons et al., 1974; 1976) and other cheeses (Bottazzi et al., 1976; Corradini et al., 1976).

As calf rennet substitute, porcine pepsin has some distinct advantages. It is considerably cheaper than other rennet substitutes (Green, 1972; 1977). In fact, the commercial success of the rennet-pepsin blends is mainly due to the low cost of pepsin advantageously reflected in the product price (Sardinas, 1976). Porcine pepsin is commercially available, and the supply is more stable than calf rennet and some rennet substitutes.

However, when used alone in cheese-making, porcine pepsin suffers from a number of disadvantages. The curd formed is not as firm as calf rennet's, and there is some loss of fat in the whey. Organoleptic quality of pepsin cheese is inferior to that of rennet cheese (Green, 1972). Furthermore, a longer ripening period is required in

cheese made with pepsin alone (Melachouris and Tuckey, 1964).

The last drawback is due to the instability of porcine pepsin under cheese-making conditions, mainly high pH's. Green and Foster (1974) observed that coagulating enzymes retained in the cheese curd contribute significantly to casein hydrolysis, and that starter enzymes and rennet are synergistic in their action on caseins and their breakdown products (Ohimiya and Sato, 1972). In spite of some controversy (O'Keefe et al., 1977), the majority of available experimental evidence indicates that porcine pepsin is almost completely denatured during cheese manufacture while at least some added calf rennet is recovered and contributes to proteolysis in cheese ripening (Green, 1972; Holmes and Ernstrom, 1973; Green and Foster, 1974).

Consistent with the above reports, the present results also show that porcine pepsin was rapidly inactivated in buffer at pH 6.5 and in milk ultrafiltrate under simulated cheese-making conditions. After carboxyl modification, however, the stability of pepsin was significantly improved. This could greatly enhance the utilization of porcine pepsin in cheese manufacture. If some active pepsin is retained in cheese curd, the ripening period could be shortened thus lowering the

production cost. In addition, some defects associated with pepsin cheese such as harder body and slow flavor development (Maragoudakis et al., 1961; Melachouris and Tuckey, 1964; Emmons et al., 1971) may also be overcome since these defects are probably attributed to a slow rate of proteolysis in the cheese.

Although the ultimate conclusion on the suitability of a milk coagulant should be derived from large scale cheese manufacture, some simple screening procedures have been described. These include non-protein nitrogen test (DeKoning, 1972), measurement of the rate of firming of the milk gel after coagulant addition (Stavlund and Kiermeier, 1973) and measuring the stability of enzyme in a simulation of the early stages of cheese-making (Green and Stackpoole, 1975). The present results indicate that the carboxyl modified pepsin has increased pH and heat stability under cheese-making conditions. It also has caseinolytic properties similar to those of the native pepsin or chymosin. The rate of syneresis and curd tension development were also similar to those observed in native pepsin and chymosin.

One drawback of the modified pepsin is the decreased milk clotting activity. However, modified 1:10,000 pepsin seems to retain higher milk clotting activity than the crystalline enzyme while the increase in stability is still

significant. The degree of loss of milk clotting activity can be controlled by the extent of modification. A loss of 50% activity would double the clotting time which should not be a critical factor in the making of cheese.

Since porcine pepsin has relatively high proteolytic activity, the active enzyme retained in the curd may lead to extensive breakdown of proteins resulting in bitterness and other textural defects. However, as the stability of porcine pepsin is dependent on the extent of carboxyl modification, the amount of active pepsin retained can be adjusted by controlling the degree of modification on the enzyme.

A principle deterrent in utilizing chemical modification in food proteins is the cost associated with proving to regulatory agencies that the products are non-toxic. Carbodiimides have been known to be toxic, but as they are just used as a coupling reagent in the reaction and are removed by dialysis, the quantity retained in the modified enzyme would be very small. Furthermore, since the clotting enzyme:milk ratio is generally low in cheese-making, and a large percentage of coagulant is lost in the whey, the concentration of carbodiimide in the cheese, even if bound to the enzyme, would be too low to be of any great significance.

The reagents used in carboxyl modification are relatively inexpensive and the reaction is simple and mild. Hence, the cost of producing carboxyl modified pepsin would not be too high, and the price of the enzyme should be at least competitive to that of the calf rennet.

The present data show the possibility of modifying the activity, specificity and physical properties of an enzyme by chemical derivatization. This could widen the scope of food-related enzymes in the food industry. Problems associated with enzyme-catalysed processes may be solved if the undesirable characteristics of the enzyme are identified and can be corrected by chemical modifications. The present finding also indicates the possibility of chemically modifying the properties of an enzyme to imitate another enzyme. It may be feasible to substitute enzymes that are expensive and/or in short supply with cheaper modified enzymes if their properties are compatible with the substituted enzymes.

CONCLUSIONS

Selective modification of carboxyl groups in porcine pepsin by water-soluble EDC and amino acid methyl esters caused significant changes in activities, specificity and physicochemical properties of the enzyme.

(1) The milk clotting activity was significantly decreased while the proteolytic activity against hemoglobin was not altered. Consequently, the milk clotting:proteolytic activity ratio was markedly decreased. The peptidase activity against APDT was decreased by about 50%.

(2) The charge density of pepsin was altered by carboxyl modification. This was shown by a decrease in relative electrophoretic mobility, a slight increase in isoelectric point and a shift in the proteolytic activity pH profile.

(3) The caseinolytic properties were not affected. However, the clotting activity against κ -casein was increased while the clotting activity against κ - α_{s1} -casein was significantly decreased. Results suggest that the drop in milk clotting activity may be attributed to a change in the charge distribution on the modified enzyme, thus hindering the interaction between pepsin and micelles.

(4) Kinetic study using dipeptide substrate shows that K_m was increased while k_{cat} was not significantly

altered. This indicates that the lowering in peptidase activity was caused by an interference with the enzyme-substrate binding process, and the catalysis of the enzyme-substrate complex was not affected.

(5) The presence of dipeptide substrates interfered with the carboxyl modification, suggesting that the modified carboxyl groups were located near the enzyme-substrate binding site.

(6) The carboxyl modification was not specific to pepsin. Modification of carboxyl groups in pepsinogen and chymosin caused similar changes in activities and properties.

(7) The modified pepsin remained reactive to two site-specific pepsin inhibitors. However, the modified enzyme was less reactive to the inactivators than the native pepsin.

(8) The stability of the modified enzyme was markedly improved in 0.05 M phosphate buffer at pH 6.5 and in milk ultrafiltrate under simulated cheese-making conditions. The thermal stability for milk clotting was also improved. The quality of the curds was not affected by the modification and was compatible with that of chymosin curds. These data suggest that the modified enzyme may be a more suitable rennet substitute than native pepsin for cheese-making.

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