Harrington, T. A., *For. Prod. J.* **19**(6), 31 (1969).
Hellyear, R. O., Lassak, E. V., *Aust. J. Chem.* **21**, 2585 (1968).
Howell, J., Jr., "Pinyon and Juniper—a Preliminary Study of Volume, Growth, and Yield", USDA Soil Conservation Service, Reg. 8 Bull. 71, For. Ser. 12, Albuquerque, NM, 1940.
Iloff, P. M., Jr., Mirov, N. T., *J. Am. Pharm. Assoc., Sci. Ed.* **43**, 738 (1954).
Joye, N. M., Jr., Lawrence, R. V., *J. Chem. Eng. Data* **12**, 279 (1967).
Lightwood Research Coordinating Council, Proceedings, 1975, Stone, R. N., Ed., Jacksonville, FL.
Lightwood Research Coordinating Council, Proceedings, 1976, Esser, M. H., Ed., Jacksonville, FL.
Lightwood Research Coordinating Council, Proceedings, 1977, Esser, M. H., Ed., Atlantic Beach, FL.
McGuire, J. R., *U.S. Dep. Agric. For. Serv., For. Res. Rep.* No. **20** (1973) (Figures cited are based on statistics for 1970).
Mirov, N. T., *J. Am. Pharm. Assoc., Sci. Ed.* **41**, 673 (1951).
Mirov, N. T., Iloff, P. M, Jr., *J. Am. Pharm. Assoc., Sci. Ed.* **45**, 629 (1956).
Mirov, N. T., *U.S. Dep. Agric. For. Serv. Tech. Bull.* No. **1239** (1961).
Mirov, N. T., Zavarin, E., Bicho, J. G., *J. Pharm. Sci.* **51**, 1131 (1962).
Snajberk, K., Zavarin, E., *Phytochemistry* **14**, 2025 (1975).
USDA, Naval Stores Handbook, Misc. Publ. 209 (1935).
Verghese, J., *Flavour Ind.* **3**, 252 (1972).
Verghese, J., *Perfum. Essent. Oil Rec.* **57**, 694 (1966).
Verghese, J., *Perfum. Essent. Oil Rec.* **58**, 638 (1967).
Verghese, J., *Perfum. Essent. Oil Rec.* **59**, 439, 876 (1968).
Verghese, J., *Perfum. Essent. Oil Rec.* **60**, 25, 271 (1969).
Wrolstad, R. E., Jennings, W. G., *J. Chromatogr.* **18**, 318 (1965).
Zavarin, E., Snajberk, K., Lee, Ch.-J., Henley, M., Mirov, N. T., *Phytochemistry* **10**, 1857 (1971).
Zavarin, E., Snajberk, K., Senter, P., *For. Sci.* **25**, 20 (1979).
Zinkel, D. F., *Appl. Polym. Symp.* **309** (1975).

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Chemical Modification of Carboxyl Groups in Porcine Pepsin

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Modification of up to 11 carboxyl groups in porcine pepsin with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and glycine methyl ester caused changes in activities, specificity, and physicochemical properties of the enzyme. The milk clotting activity was markedly decreased to 10%, while the proteolytic activity was not affected. The decrease in the peptidase activity was about 50%. The charge density of pepsin decreased upon modification, as shown in a decrease of relative electrophoretic mobility and in a shift of pH optimum from 2 to 3.5. Kinetic studies showed that K_m was increased, while k_{cat} was not significantly affected. The presence of dipeptide substrates interfered with the modification. The modified pepsin remained reactive to two site-specific pepsin inhibitors. These effects of carboxyl modification were not unique to pepsin; modification of carboxyl groups caused similar changes in the activities and properties of pepsinogen and chymosin. The stability of the modified pepsin near neutral pH was considerably improved, suggesting that the modified enzyme may be a more suitable rennet substitute than native pepsin in cheese-making.

Porcine pepsin (EC 3.4.23.1) has been used as a calf rennet substitute in cheese making (Bottazzi et al., 1976; Carbone and Emaldi, 1976; Phelan, 1973), but the use of pepsin alone is considered unsuitable (Green, 1972; Sardinas, 1972). The objective of this study was to change the activities, specificity, and physicochemical properties of pepsin by chemical modification, hoping that these alterations could enhance the potential utilization of pepsin in the manufacture of cheese. Carboxyl modification with water-soluble carbodiimides (Hoare and Koshland, 1967) has been found to change the properties of some enzymes (Matyash et al., 1973; Swaisgood and Natake, 1973). In the present study, the carboxyl groups in pepsin were modified and the changes in enzymatic activities, kinetics, and some physicochemical properties were investigated. The role played by the nonessential carboxyl groups of pepsin in its function was also assessed.

EXPERIMENTAL SECTION

Materials. Porcine pepsin (2× crystallized), pepsinogen, and chymosin were purchased from Sigma Chemical Co., St. Louis, MO. Pepsin was homogeneous as indicated by gel electrophoresis and DEAE-cellulose column chromatography at pH 4.2 and was used without further purification. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), *N*-acetyl-L-(phenylalanyl)-L-diiodotyrosine (APDT), *N*-acetyl-D-(phenylalanyl)-L-tyrosine, *N*-(carbobenzoxy)-L-glutamyl-L-tyrosine, and the methyl esters of arginine, leucine, lysine, tyrosine, and tryptophan were also products of Sigma Chemical Co. Glycine methyl ester was purchased from Aldrich Chemical Co., Montreal, P.Q. Two pepsin inhibitors, 1,2-epoxy-3-(*p*-nitrophenoxy)propane and bromophenacyl bromide, were purchased from Eastman Kodak Co., Rochester, NY.

Modification of Carboxyl Groups. The carboxyl groups in pepsin were modified by the carbodiimide-promoted amide formation. The method employed was essentially that of Hoare and Koshland (1967) except that dissociating agents were omitted and sufficiently lower concentrations of carbodiimide and nucleophile were used to achieve limited modification. The enzyme (10 mg/mL) and nucleophile were dissolved in distilled water, and the pH of the solution was adjusted to 5.5 with 0.1 N NaOH. The carbodiimide EDC was added as a solid to obtain the desired concentration. The reaction mixture was stirred

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Table I. Effect of Carboxyl Modification on Pepsin Activities

EDC		Gly methyl ester		no. of COOH grp modified/mol of pepsin	milk clotting activity, % control	proteolytic activity, % control		peptidase activity, % control
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	100
10	33	25	87	2.8	27	88	210	55
10	33	50	174	5.2	17	94	340	50
10	33	100	348	8.6	13	105	330	44
10	33	200	706	11.2	9	90	340	40
0	0	50	174	0	100	100	100	100
5	16.5	50	174	1.0	60	100	120	100

continuously at room temperature for 90 min. The pH of the reaction mixture was kept constant at 5.5 by automatic addition of 0.1 N HCl, using a Radiometer type TTTlc titrator (Copenhagen, Denmark). The reaction was terminated by the addition of excess 3 M sodium acetate buffer at pH 5.5. The residual reagents were removed by exhaustive dialysis against distilled water for 48 h at 4 °C, and the modified protein was recovered by lyophilization. The extent of carboxyl modification was determined by amino acid analysis.

Determination of Pepsin Activities. The milk clotting activity of pepsin and chymosin was determined by the method of Berridge (1945). A modified method of Foltmann (1970) was also used occasionally for checking. Both procedures were found to give similar results with good repeatability. One unit of milk clotting activity is the amount of enzyme that would clot 10 mL of reconstituted skim milk in 100 s at 30 °C.

The proteolytic activity of pepsin was determined at pH 2.0 by the method of Anson (1938). One unit of proteolytic activity is defined as the amount of enzyme that produces trichloroacetic acid soluble peptides, yielding an absorbance change at 280 nm of 0.001/min at 37 °C.

The peptidase activity of pepsin was determined at pH 2.0 by the assay method of Jackson et al. (1965) with modifications as described by Ryle (1970), using *N*-acetyl-L-phenylalanyl-L-diiodotyrosine as substrate. One unit of peptidase activity is defined as the quantity of enzyme which liberates 1 μmol of diiodotyrosine/min at 37 °C.

To determine pepsinogen activities, the zymogen was activated by overnight incubation at room temperature at a concentration of 1 mg/mL in 0.01 M KCl–HCl, pH 2.0.

Agarose Gel Electrophoresis. Electrophoresis of enzymes was carried out with the agarose film cassette system of Analytical Chemists, Inc., Palo Alto, CA. In calculating the relative electrophoretic mobility of the modified enzymes, the native enzymes were assumed to have a R_f value of 1.00.

Determination of K_m and k_{cat}. The initial velocity (v_o) of the pepsin-catalyzed hydrolysis of APDT was determined at different substrate concentrations. The Michaelis constant, K_m, was determined by the Lineweaver–Burk plots. The molecular activity coefficient, k_{cat}, was calculated from the Michaelis–Menten equation:

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

where [E] is the total enzyme concentration and [S] is the substrate concentration at zero time. K_m and k_{cat} were determined at both pH 2.0 and 4.5.

Determination of Pepsin Stability Near Neutral pH. The pH stability of pepsin in dilute buffer was determined by the method described by Lowenstein (1974). The stability of pepsin in milk ultrafiltrates under simulated cheese-making conditions was also studied, using the method described by Green (1972). Reconstituted milk from skim milk powder was passed through an ultrafiltration cell (Model 52, Amicon Corp., Lexington, MA) equipped with a PM 10 Diaflo membrane. The filtrate collected was stored at 4 °C and used within 2 days after preparation.

RESULTS

Effect of Carboxyl Modification of Pepsin Activities. Table I shows the effect of carboxyl modification on milk clotting and proteolytic and peptidase activities of pepsin. The number of carboxyl groups modified increased as the concentration of nucleophile was increased. The concentration of EDC was less critical in affecting the extent of modification, but EDC concentrations less than 10 mM (33-fold excess to pepsin) were found to be ineffective in modifying pepsin. In subsequent experiments, unless specified otherwise, the concentrations of reagents used were 33-fold excess of EDC and 174-fold excess of glycine methyl ester, corresponding to an incorporation of 5.2 mol of nucleophile per mol of pepsin.

The milk clotting activity decreased 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 pepsin was treated with 10 mM EDC in the absence of nucleophile, the milk clotting activity was found to decrease by about 35%.

The proteolytic activity of pepsin at pH 2.0 was found to drop slightly after modification. Even with extensive modification (11.2 mol of nucleophiles incorporated), the enzyme still retained 90% of its proteolytic activity. When the proteolytic activity was measured at pH 3.5, the modified enzyme showed a marked increase in activity, suggesting a shift in pH profile. The peptidase activity of pepsin against APDT at pH 2.0 decreased by about 40–60% upon carboxyl modification.

Effect of Carboxyl Modification on pH Profiles. The pH activity curves for milk clotting and hydrolysis of APDT were not significantly altered by carboxyl modification. The proteolytic pH profile, however, was affected by modification. The native pepsin had a pH optimum at about 2.0 and the proteolytic activity decreased sharply toward higher pH (Figure 1). After modification the pH optimum was shifted to about 3.5.

Electrophoretic Mobility of Native and Carboxyl Modified Pepsins. Both the native and modified enzymes appeared as one band on the agarose film. The electrophoretic mobility decreased progressively with increase in the extent of carboxyl modification (Figure 2).

Carboxyl Modification of Pepsin Using 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 (Table II). All nucleophiles were

Table II. Carboxyl Modification of Pepsin, Using Different Amino Acid Methyl Esters [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 min]

methyl ester	no. of COOH grp modified/ mol of pepsin	milk clotting activity, % control	proteolytic activity, % control, pH 2.0	proteolytic activity, % control, pH 3.5	R_f[a]
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
Trp	3.5	4	40	25	0.70
Gly	5.2	17	94	340	0.56

[a] Native pepsin was assumed to have a R_f value of 1.00.

Table III. Kinetics of the Hydrolysis of N-Acetyl-L-phenylalanyl-L-diiodotyrosine by the Native and Carboxyl-Modified Pepsins

enzyme	concn, μM	pH	K_m, M × 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

found to incorporate into pepsin, but the extent of modification was variable. The milk clotting activities of all modified enzymes were markedly reduced, particularly those modified with tyrosine and tryptophan methyl esters. At pH 2.0, the proteolytic activity of pepsin modified with arginine and lysine methyl esters was not changed, while pepsins treated with leucine, tyrosine, and tryptophan methyl esters showed a decrease in activity. The proteolytic activity at pH 3.5 was significantly increased in most modified enzymes. However, tyrosine methyl ester treated pepsin did not show a change in activity, while the tryptophan derivative showed a marked decrease in activity at pH 3.5. The relative electrophoretic mobility of all modified enzymes decreased as compared to the native enzyme, particularly pepsin modified with cationic amines, e.g., arginine and lysine methyl esters.

Effect of Carboxyl Modification on K_m and k_{cat}. K_m and k_{cat} values comparable to the published data (Jackson et al., 1965) were obtained for native pepsin. After modification, K_m increased significantly at both pH 2.0 and 4.5, while k_{cat} was not markedly altered (Table III).

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-(carbobenzoxy)-L-glutamyl-L-tyrosine], and a nonsubstrate [N-acetyl-D-(phenylalanyl)-L-tyrosine]. The extent of modification, the milk clotting activity and the electrophoretic mobility of pepsins after treatments were determined (Table IV). In the presence of a sensitive substrate, the extent of modification was significantly decreased as compared to pepsin modified in the absence of substrate. The milk clotting activity and electrophoretic mobility were only moderately lowered in the presence of

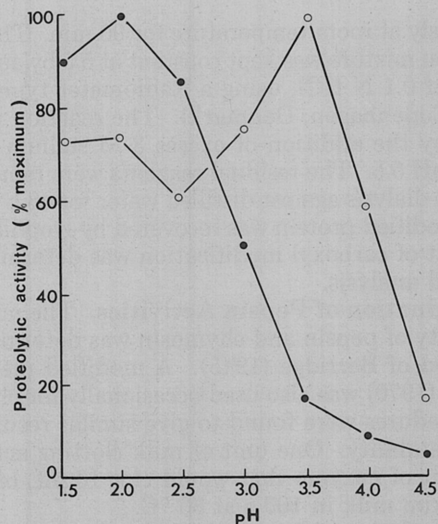

Figure 1. Proteolytic pH profiles of pepsin: native pepsin (●), carboxyl-modified pepsin (○). The enzyme (10 mg/mL) was modified with 33-fold excess of EDC and 174-fold excess of glycine methyl ester at pH 5.5 for 90 min.

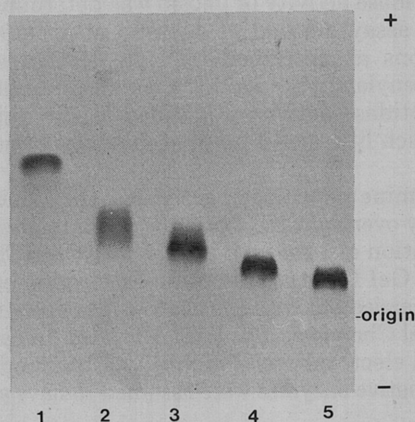

Figure 2. Agarose gel electrophoretic patterns of the native and carboxyl-modified pepsins. Electrophoresis was carried out in 0.05 M barbital buffer (pH 8.6) containing 0.035% EDTA. The time of electrophoresis was 40 min. Number of carboxyl groups modified/mole of pepsin: 1/0 (native pepsin), 2/5.2, 3/8.7, 4/11.2, 5/15.6. Amount of protein applied: 5 μg/sample.

Table IV. Effect of Dipeptides on the Carboxyl Modification of Pepsin

addition	concn, mM	no. of COOH grp modified	milk clotting activ, % control	R_f
1. none		0	100	1.00
2. EDC + Gly methyl ester	10 50	5.2	17	0.56
3. 2 + N-acetyl-L-phenylalanyl-L-diiodotyrosine	0.3	2.3	50	0.90
4. 2 + N-carbobenzoxy-L-glutamyl-L-tyrosine	2	3.6	35	0.77
5. 2 + N-acetyl-D-phenylalanyl-L-tyrosine	2	4.8	22	0.65

Table V. Response of the Native and Carboxyl-Modified Pepsins to Inhibitors [The Conditions of Incubation of Pepsin with the Inhibitors Were Those Described by Chen and Tang (1972) and Erlanger (1965)]

enzyme	inhibitor	concn	proteolytic activity, % retained
pepsin	1,2-epoxy-3-(*p*-nitrophenoxy)propane	1 mg/mg of protein	10
COOH-modified pepsin	1,2-epoxy-3-(*p*-nitrophenoxy)propane	1 mg/mg of protein	27
pepsin	bromophenacyl bromide	200 μM	30
COOH-modified pepsin	bromophenacyl bromide	200 μM	65

Table VI. Effect of Carboxyl Modification on the Activities of Pepsin, Pepsinogen, and Chymosin [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 min]

enzyme	no. of COOH grp modified	enzyme activity, % control: milk clotting, pH 6.3	proteolytic pH 2.0	proteolytic pH 3.5
pepsin	11.2	9	90	340
pepsinogen	12.1	30	70	450
chymosin	13.0	15	50	50

APDT. In the presence of a poor substrate and a nonsubstrate dipeptide, pepsin was also modified, but to a lesser extent than the fully modified control.

Response of Native and Modified Pepsins to Inhibitors. The reactions of native and carboxyl modified pepsins with inhibitors were studied. 1,2-Epoxy-3-(*p*-nitrophenoxy)propane, a substrate-like epoxide inactivator which blocks Asp-32 in pepsin (Chen and Tang, 1972), and bromophenacyl bromide, an inhibitor which reacts with a carboxyl group in pepsin not directly involved in catalysis (Erlanger et al., 1965), were used. Results show that after 120 h of incubation at room temperature with 1,2-epoxy-3-(*p*-nitrophenoxy)propane, native pepsin retained only 10% of the proteolytic activity. The modified enzyme, on the other hand, retained 30% of its activity. Bromophenacyl bromide caused a loss of 70% activity to native pepsin, and only 35% to the modified enzyme (Table V).

Specificity of Carboxyl Modification. To investigate whether the carboxyl modification by the carbodiimide reaction was specific to pepsin, pepsinogen and chymosin were modified under identical conditions. Results show that pepsinogen and chymosin were modified to about the same extent as pepsin, with a concomitant decrease in the milk clotting activity. At pH 2.0, the proteolytic activity of both the modified pepsinogen and chymosin decreased. At pH 3.5, the modified pepsinogen showed a significant increase in proteolytic activity, while the activity of the modified chymosin was found to decrease by about 50% (Table VI). The relative electrophoretic mobilities of pepsinogen and chymosin significantly decreased after modification.

Effect of Modification on Pepsin Stability Near Neutral pH. Figure 3 shows the stability curves of native and carboxyl-modified pepsins in dilute phosphate buffer at pH 6.5. The milk clotting activity of native pepsin decreased rapidly, following a first-order kinetic with a half-life ($t_{1/2}$) for inactivation of 15 min. Less than 10% activity was retained after 1 h of incubation. With mild modification (87-fold excess of nucleophile), the stability was markedly improved, and the enzyme retained about 20% of milk clotting activity after incubation. When pepsin was more extensively modified (174-fold excess of nucleophile), the stability was further improved, and the loss in activity was only about 50% after 4 h of incubation.

Figure 4 shows the stability curves of native and modified pepsins in milk ultrafiltrate at 30 °C with progressive adjustment of pH according to the method of Green (1972). 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 h, with a loss of 80% in activity after incubation. After modification, the stability of the enzyme was markedly improved. The rate of decline in activity was much more gradual when compared to the control, and the decrease in activity was about 40%.

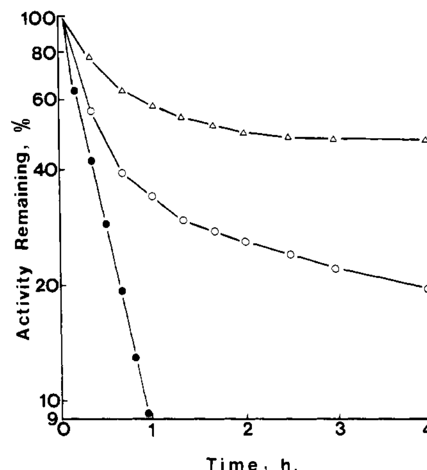

Figure 3. Stability of native and carboxyl-modified pepsins in 0.05 M phosphate buffer, pH 6.5: (●) native pepsin, (○) pepsin modified with 87-fold excess of nucleophile, (△) pepsin modified with 174-fold excess of nucleophile.

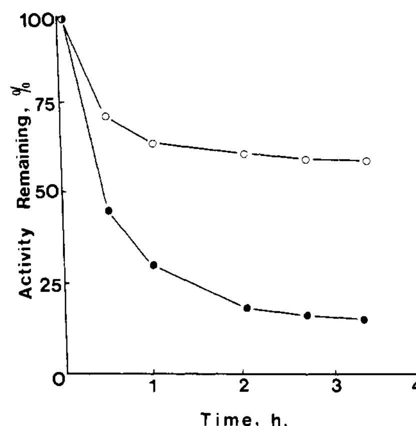

Figure 4. Stability of native and carboxyl-modified pepsins in milk ultrafiltrate under simulated cheese-making conditions: (●) native pepsin, (○) carboxyl-modified pepsin.

DISCUSSION

The data presented in this study show that limited modification of pepsin caused profound changes in enzymatic activities and properties. The retardation in electrophoretic mobility on agarose gel is an indication of a decrease in the net negative charge on the modified enzyme. Due to the low gel concentration and large pore size of agarose used, the molecular sieving effect, prominent

in starch and polyacrylamide gels, is minimal (Shaw, 1969). Gel filtration chromatography of the 11.2 carboxyl groups modified pepsin on Sephadex G-200 (Superfine) with 0.1 M sodium acetate buffer (pH 4.5) confirmed that the change in molecular size was insignificant, as the modified enzyme was eluted at the same elution volume as the native pepsin. This result also indicates that there was no aggregation of modified pepsin molecules. Hence, the decrease in electrophoretic mobility after carboxyl modification is mainly attributed to the reduced negative charge on the modified enzyme.

Presumably, the decrease in negative charge on pepsin was not only a result of the blocking of carboxyl groups with nucleophile but also 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 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide metho-*p*-toluenesulfonate (CMC) alone contained two–three residues of labeled carbodiimide. In this study, pepsin treated with 10 mM EDC in the absence of nucleophile had a R_f value of 0.89, indicating that the contribution of carbodiimide to the drop in net negative charge was significant.

The shift in the pH optimum also suggests a change in the charge distribution on the modified pepsin. Similar effects had been observed in some immobilized enzymes which were ascribed to the "microenvironment" 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 charge distribution on the enzyme molecules. Consequently, the pH in the domain of the enzymes would be different from the external bulk solution, creating an apparent shift in pH profile (Goldstein, 1970). Since the nucleophile (glycine methyl ester) attached to the pepsin in this study is of small molecular size in comparison to the carriers used in enzyme immobilization, it is unlikely that the shift in pH profile was due to the "microenvironment" effect; it is probably related to changes in the charge distribution on the modified enzyme.

The pH profile of the modified pepsin showed a shoulder at pH 2 (Figure 1). Since electrophoresis yielded only one band for the modified pepsin, the shoulder may not be due to incomplete modification, but may possibly be attributed to a heterogeneous population of modified enzymes resulting from modification at different sites on pepsin.

The dramatic drop in the milk clotting activity after modification could be due to either a blocking of carboxyl residues essential for activity or a change in the charge density of the enzyme, thus hindering the interaction with its substrate caseinate micelles. Since the proteolytic activity of the modified pepsin was not significantly decreased, it is unlikely that the carboxyl groups modified are involved in the enzymatic breakdown of casein, the first step in the coagulation of milk mediated by a proteinase. Pepsin treated with EDC alone showed a slight drop in the milk clotting activity and a lower electrophoretic mobility, suggesting that the incorporation of positively charged carbodiimide on pepsin might have caused changes in the charge distribution of the enzyme and a subsequent loss in milk clotting activity.

The present result on proteolytic activity is in contrast to that reported by Matyash et al. (1973), who showed a decrease of about 40% in the activity against hemoglobin in pepsin modified with CMC and a colored amine, although only one nucleophile was incorporated into the enzyme. One of the carboxyl groups modified by the CMC-colored amine system, Asp-243, is situated near the binding cleft of pepsin (Andreeva et al., 1976). Since two hydrophobic sites are located near the active center (Andreeva et al., 1976) and the colored amine is strongly hydrophobic, it might have greater tendency to bind to these hydrophobic sites than to glycine methyl ester and then react with carboxyl groups near the active center, thereby causing partial loss in activity.

Due to a dramatic decrease in milk clotting activity without significant change in proteolytic activity, the milk clotting/proteolytic activity ratio, an index of the specificity of proteases (Ernstrom, 1974), was markedly decreased in the modified pepsin.

The drop in peptidase activity could be attributed to a decrease in the affinity of the enzyme to the dipeptide substrate, as indicated by an increase in K_m. The fact that k_{cat} was not significantly changed suggested that the catalysis of the enzyme–substrate complex was unaffected. A change in kinetic parameters was also reported in arginine modified pepsin (Kitson and Knowles, 1971). However, in this modified pepsin, K_m did not change, while k_{cat} was significantly lowered, indicating that the enzyme–substrate binding process was unaffected and the inactivation took place during catalysis.

The variation in the response of pepsin to different amino acid methyl esters could be due to difference in solubility. Incorporation of hydrophobic amino acids such as tyrosine and tryptophan into pepsin might have decreased the solubility of the enzyme, leading to an apparent drop in activity. Alternatively, the loss in both milk clotting and proteolytic activities could be due to a greater affinity of the hydrophobic methyl esters to the hydrophobic binding sites, with subsequent modification of carboxyl groups close to the active center of pepsin.

Present data show that the effects of carboxyl modification on pepsin activity and physicochemical properties were less pronounced in the presence of dipeptide substrates. This suggests that the binding of substrates to the enzyme protects the carboxyl groups in the domain of the enzyme–substrate binding site. Conformational changes resulting from enzyme–substrate interaction may also interfere with subsequent modification. The fact that even nonsubstrate peptides hindered carboxyl modification suggests that the dipeptides react with either carboiimide or nucleophile, such that less reagents are available for pepsin modification.

Modified pepsin remained reactive to site-specific inhibitors, confirming that the carboxyl groups modified were not the active-site residues. However, the modified pepsin was less reactive to the inactivators than the native enzyme with a larger K_i. This suggests that the two carboxyl groups reactive to the inhibitors were protected in the modified pepsin, probably as a result of a conformational change or steric hindrance.

The present data show that the carbodiimide reaction is not specific to pepsin. Both the pepsin precursor and chymosin, another acid protease, were modified to show similar responses. In contrast, 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 modified zymogen and fungal protease retained 100% of their proteolytic activities. This indicates that the CMC-colored amine system is more selective than EDC–glycine methyl ester in modifying the pepsin carboxyl groups.

Our results indicate that the stability of pepsin near neutral pH was markedly improved by carboxyl modification. Porcine pepsin is rapidly denatured at pH 7.0 and

30 °C (O'Leary and Fox, 1974), probably due to an electrostatic expansion of the negatively charged polypeptide chains (Lowenstein, 1974). The decrease in net negative charge on the carboxyl-modified pepsin molecule (Figure 2) may lower the extent of electrostatic expansion and subsequent denaturation. An improvement of enzyme stability was also reported in pepsin covalently bound to a soluble polyanionic carrier, ethylene maleic anhydride, and was attributed to different environmental states of the native and modified enzymes (Lowenstein, 1974).

The present results show that native pepsin was inactivated rapidly under simulated cheese-making conditions. This is consistent with the findings of Green (1972) that crude pepsin was almost completely denatured in milk dialysate under simulated cheese-making conditions, while rennet was fairly stable, retaining about 60% of its activity. In another report, active enzyme was not recovered from Cheddar curd made by porcine pepsin, whereas 5% of the added calf rennet was recovered (Holmes and Ernstrom, 1977).

The poor quality of cheese made with pepsin has been attributed to the instability of pepsin near neutral pH. 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, proteolysis would be almost entirely dependent on starter activity (Green, 1972; Lawrence et al., 1972). Hence, cheese made with pepsin alone requires a longer aging period and develops flavor slowly (Melachouris and Tuckey, 1964; Emmons et al., 1971; Green, 1972). An increase in stability under simulated cheese-making conditions would make pepsin a more effective milk coagulant in cheese manufacture. A larger amount of active enzyme would be retained in the curd to aid ripening, thus reducing the production cost. For practical application, carboxyl-modified crude pepsin (1:10 000), with improved stability without losing the milk clotting activity more than 50% (data will be published elsewhere), would be a better coagulant than the modified crystalline enzyme.

The results presented in this paper show that the carboxyl groups modified by EDC and glycine methyl ester are not essential for pepsin activity. However, changes in the charge distribution on the enzyme may affect some pepsin functions such as milk clotting and the hydrolysis of dipeptide substrates, possibly through an interference with the enzyme–substrate binding process as shown by an increase in the K_m values upon carboxyl modification. The changes in the charge distribution may also alter some physicochemical properties of pepsin, such as enzyme stability and pH optimum for proteolysis.

LITERATURE CITED

Andreeva, N. S., Fedorov, A. A., Gushchina, A. E., Shutskever, N. E., Riskulov, R. R., Vol'nova, T. V., *Dokl. Biochem. (Engl. Transl.)* **228**, 219 (1976).
Anson, M. L., *J. Gen. Physiol.* **22**, 79 (1938).
Berridge, N. J., *Biochem. J.* **39**, 179 (1945).
Bottazzi, V., Corradini, C., Battistotti, B., *Sci. Tec. Latt.-Casearia* **25**, 43 (1974); *Dairy Sci. Abstr.* **38**, 119 (1976).
Carbone, E., Emaldi, G. C., *Sci. Tec. Latt.-Casearia* **25**, 95 (1974); *Dairy Sci. Abstr.* **38**, 50 (1976).
Chen, K. C. S., Tang, J., *J. Biol. Chem.* **247**, 2566 (1972).
Emmons, D. B., Petrasovitis, A., Gillan, R. H., Bain, J. M., *Can. Inst. Food Sci. Technol. J.* **4**, 31 (1971).
Erlanger, B. F., Vratsanos, S. M., Wassermann, N., Cooper, A. G., *J. Biol. Chem.* **240**, 3447 (1965).
Ernstrom, C. A. in "Fundamentals of Dairy Chemistry", 2nd ed, Webb, B. H., Johnson, A. H., Alford, J. A., Eds., Avi, Westport, CT, 1974, p 662.
Foltmann, B., *Methods Enzymol.* **19**, 421 (1970).
Goldstein, L., *Methods Enzymol.* **19**, 935 (1970).
Green, M. L., *J. Dairy Res.* **39**, 261 (1972).
Hoare, D. G., Koshland, D. E., *J. Biol. Chem.* **242**, 2477 (1967).
Holmes, D. G., Ernstrom, C. A., *J. Dairy Sci.* **60**, 862 (1977).
Jackson, W. T., Schlamowitz, M., Shaw, A., *Biochemistry* **4**, 1537 (1965).
Kitson, T. M., Knowles, J. R., *FEBS Lett.* **16**, 337 (1971).
Lawrence, R. C., Creamer, L. K., Gilles, J., Martley, F. G., *N.Z. J. Dairy Sci. Technol.* **7**, 32 (1972).
Lowenstein, H., *Acta Chem. Scand., Ser. B* **28**, 1098 (1974).
Matyash, L. F., Ogloblina, O. G., Stepanov, V. M., *Eur. J. Biochem.* **35**, 540 (1973).
Melachouris, N. P., Tuckey, S. L., *J. Dairy Sci.* **47**, 1 (1964).
O'Leary, P. A., Fox, P. F., *J. Dairy Res.* **41**, 381 (1974).
Phelan, J. A., *Dairy Ind.* **38**, 418 (1973).
Ryle, A. P., *Methods Enzymol.* **19**, 316 (1970).
Sardinas, J. L., *Adv. Appl. Microbiol.* **15**, 39 (1972).
Shaw, D. J., "Electrophoresis", Academic Press, New York, 1969.
Silman, I. H., Katchalski, E., *Annu. Rev. Biochem.* **35**, 873 (1968).
Swaisgood, H., Natake, M., *J. Biochem. (Tokyo)* **74**, 77 (1973).

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Fatty Acid Biogenesis in Ripening Mango (*Mangifera indica* L. var. Alphonso)

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The biogenesis of fatty acids in the ripening mango (var. Alphonso) was investigated with the aid of [2-^{14}C]acetate and [1-^{14}C]palmitic acid. It was observed that [2-^{14}C]acetate incorporation was maximal into palmitic acid and to a lesser extent in palmitoleic acid, while the radioactivity of [1-^{14}C]palmitic acid could be recovered essentially in the hydroxy fatty acids. The contribution of C-16 fatty acids to the formation of lactones in ripe Alphonso mango was discussed.

Lipid components in fruits, though occurring in minor amounts, are presumed to contribute to characteristic aroma and flavor during ripening. These are essentially considered as precursors for various volatile odorous principles of fruits. Several lactones identified in peaches and apricots (Jennings and Sevenants, 1964), as well as esters in bananas (Tressl and Drawert, 1973), are believed to be of lipid origin. Ripening of mango fruit (var. Alphonso) has been shown to be accompanied by an increase in glyceride content of the pulp, followed by changes in component fatty acids, particularly with respect to the ratio of palmitic to palmitoleic acids, and fruit aroma

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