ARTICLES

Reduction of Negative Charge in the Aspartyl Proteinase from the Fungus *Mucor miehei* by Chemical Modification of Carboxyl Groups: Effect on Structure–Function

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Decreased negative charge in the aspartyl proteinase from *Mucor miehei* (MMP) by modification of carboxyl groups with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide and different nucleophiles (methyl esters of glycine, leucine, arginine, and tryptophan) reduced proteolytic and milk-clotting activity an average of 24 and 93%, respectively. A shift in the pH–activity optimum from pH 5.0 to pH 3.0 or 3.5 (depending on nucleophile), increased pH–stability, and generally lower temperature–activity optimum and range were also consequences of modification. Relative to native enzyme, enthalpy of denaturation values and peak denaturation temperatures, determined by differential scanning calorimetry, were lower only for arginine and tryptophan methyl ester-modified MMP; the kinetic and thermodynamic parameters activation energy of denaturation and change in free energy, respectively, indicated compromised stability of all carboxyl-modified derivatives. Changes in functional properties of modified MMP were associated with changes in tertiary structure as evidenced by decreased near-UV CD spectral intensity. No change in the proportions of secondary structure fractions was observed. Results from this study indicated that the reduction of negative charge via carboxyl modification was not a viable means for increasing the cheese-making potential of MMP.

INTRODUCTION

Several aspartyl proteinases have been used as calf rennet substitutes to coagulate milk for cheese-making, e.g., porcine and bovine pepsins and proteinases from the fungi *Mucor miehei*, *M. pusillus*, and *Endothia parasitica* (Birkkjaer and Johnk, 1985). Of the possible alternate milk-clotting enzymes, *M. miehei* proteinase (MMP) is the most commonly used because of its comparatively superior cheese-making properties. However, it is unsuitable for the production of some types of cheese because of its high thermostability which results in texture and flavor defects in the final product (Garnot, 1985).

In addition to thermostability, the ratio of milk-clotting activity to proteolytic activity (MC/PA) is an important determinant in the selection of a chymosin substitute. Enzymes suitable for cheese-making should combine high milk-clotting activity with low proteolytic activity. Previous studies have reported a MC/PA for chymosin that was substantially greater than that for the aspartyl proteinase from *M. miehei* (Yada and Nakai, 1986a; Smith and Yada, 1991). For MMP to be considered a suitable chymosin replacement for the manufacture of all types of cheese, increased MC/PA and reduced thermostability must be achieved, e.g., through chemical modification or site-directed mutagenesis.

Of the methods available for modification of enzyme activity and/or specificity, controlled chemical modification is perhaps the most facile. Chemical modification of enzymes has been widely used in the study of their catalytic mechanisms, stability improvements, and immobilization. Although the chemical approach to enzyme modification is compromised to various degrees by a general lack of specificity, such an approach may aid in the identification of groups essential for catalysis or particular functional properties that can subsequently be targeted for site-specific mutations to yield improved/altered properties. Alternately, chemical modification can be performed to protect specific groups, i.e., nontarget groups, against (further) modification, thereby improving the selectivity of subsequent modification procedures. Planas and Kirsch (1990) recently reported on a sequential protection–modification strategy to selectively derivatize a specific cysteine residue in aspartate aminotransferase from the K258C mutant of *Escherichia coli.*

More directly, nonselective chemical modification, i.e., modification of a specific type of amino acid residue, may yield enzymes with desired properties, thereby increasing their potential application. Ma and Nakai (1980) reported that chemical modification of carboxyl groups in porcine pepsin improved the stability of the enzyme near neutral pH.

Yada and Nakai (1986a) proposed that the high MC/PA of chymosin may be related to its high hydrophobicity and low charge. To date, no studies have been reported on the structure–function of MMP in which reduction of negative charge has been effected. The present study was, therefore, undertaken to investigate the effect(s) of reduction of negative charge by nonspecific chemical modification of carboxyl groups on the physicochemical properties and structure of MMP.

MATERIALS AND METHODS

Materials. MMP was obtained as a lyophilized preparation (rennet type II, mutated strain CBS 370.65, lot 23F-0001, Sigma Chemical Co., St. Louis, MO). 1-Ethyl-3-[3-(dimethylamino)-propyl]carbodiimide (EDC), methyl esters of glycine, leucine, arginine, and tryptophan, and guanidine hydrochloride were also products of Sigma. Guanidine hydrochloride (GdnHCl) was used without further purification. Stock solutions of GdnHCl were

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prepared in 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 5.5); concentrations were determined from refractive index measurements (Nozaki, 1972).

Enzyme Purification. MMP was purified using gel permeation chromatography (Sephadex G-25, Pharmacia, Uppsala, Sweden) and horizontal isoelectric focusing (Smith and Yada, 1991).

Modification of Carboxyl Groups. Carboxyl groups in purified MMP were modified according to the carbodiimide-mediated amide formation procedure of Hoare and Koshland (1967), as modified by Ma and Nakai (1980). The procedure involved activation of protein carboxyl groups, i.e., carboxyl side chains of aspartic and glutamic acids, by water-soluble EDC subsequent to nucleophilic attack by an amino acid methyl ester.

Purified MMP (50 mg) was dissolved in 5 mL of distilled water and the pH adjusted to 5.5 with 0.1 N NaOH. EDC and nucleophile were added to concentrations between 0 and 20 and 0 and 200 mM, respectively. The reaction mixture was stirred continuously at room temperature (25 °C) for up to 240 min while the pH was maintained at 5.5 ± 0.2. The reaction was terminated by the addition of excess (25 mL) 3 M sodium acetate buffer (pH 5.5); final sample volume was approximately 35 mL. Residual reagents were removed by exhaustive dialysis (molecular weight cutoff of 12 000–14 000) against distilled water (10 changes at 3 L/change) for 48 h at 4 °C. Modified MMP was recovered by lyophilization and the extent of carboxyl modification determined by amino acid analysis. All subsequent analyses were performed at least in triplicate on two MMP samples modified independently using each of the amino acid methyl esters.

Protein Concentration. The protein concentration of 1 mg/mL solutions of native and carboxyl-modified MMP was determined using the molar extinction coefficient of 1.18×10^{-4} at 280 nm (Ottesen and Rickert, 1970) and was confirmed using the modified Lowry method (Hartree, 1972) with bovine serum albumin as standard.

Physicochemical Characterization. The protocols used for determination of proteolytic activity (acid-denatured hemoglobin substrate), milk-clotting activity (Berridge substrate; Berridge, 1952), susceptibility to pepstatin A inhibition, and pH– and temperature–activity optimum and range of native and carboxyl-modified MMP were as previously reported (Smith and Yada, 1991). Amino acid analysis was conducted similarly to the procedure of Smith and Yada (1991) except that 6 M HCl was used. Tryptophan content was estimated spectrophotometrically (Edelhoch, 1967). Agarose gel electrophoresis was carried out on an Atto electrophoretic unit (Atto Corp., Tokyo) with ultrapure DNA grade agarose (Bio-Rad Laboratories, Richmond, CA) made up to 1% (w/v) in 50 mM sodium barbital buffer (pH 8.6). Coomassie Brilliant Blue G-250 stained gels were scanned with a Zenith Soft-Laser densitometer (Model SLR-504-XL, Biomed Instruments, Fullerton, CA) to determine relative electrophoretic mobility of the bands; native MMP was assigned a relative value of 1.00. Agarose gel electrophoresis was performed since the use of low gel concentration (e.g., 1%) and resultant large pore size minimized molecular seiving and allowed separation of proteins based primarily on charge (Shaw, 1969; Ma and Nakai, 1980).

Differential scanning calorimetry (DSC) measurements were made on a Du Pont differential scanning calorimeter Model 2910 and Thermal Analyst 2000 System (E. I. du Pont de Nemours and Co., Wilmington, DE) at a heating rate of 2 °C/min from 25 to 100 °C. The instrument was calibrated with indium at the specified heating rate. Protein samples were made up in 20 mM MES buffer (pH 5.5) to concentrations between 6 and 8% (w/v) 30 min prior to thermal analyses. Sample volumes of 15 μL were hermetically sealed in aluminum pans; a pan containing the same volume of MES buffer served as the control. The enthalpy of denaturation (ΔH_D), determined from the area under DSC endotherms, was used as a quantitative measure of proteinase thermostability (Donovan and Ross, 1973; Donovan and Beardslee, 1975). Areas were calculated by the Du Pont Thermal Analyst 2000 General Utilities V4.0 program (Du Pont) using a linear baseline option. Peak denaturation temperature (T_D), defined as the temperature of maximum rate of heat flow, was also determined.

Kinetic parameters of proteinase denaturation were also determined by DSC; sample preparation was as outlined above. The rate constant (k_T) for the nth order reaction was calculated according to

$$k_T = (1 - \alpha)^n / r_T \qquad (1)$$

where α (the fraction reacted) was the ratio of peak area at temperature T to the total area and where r_T (rate of heat flow) was the vertical displacement from the baseline at temperature T (Borchardt and Daniels, 1957; Biliaderis, 1983). First-order reaction kinetics have previously been demonstrated for the thermal denaturation of aspartyl proteinases (Hyslop et al., 1979; Brown and Yada, 1991); therefore, $n = 1$ was used. Kinetic stability was indexed by the activation energy E_a of denaturation, which was calculated from values of k_T at several temperatures using the Arrhenius equation:

$$k = A^{-E_a/RT} \qquad (2)$$

Thermodynamic analyses were performed by equilibrating native and carboxyl-modified samples (0.25 mg/mL) in incremental concentrations of GdnHCl (pH 5.5) for 4 h at 4 °C subsequent to absorbance measurement at 257 and 287.5 nm (identified as critical wavelengths through difference spectroscopy; results not shown). The fraction native, f_N, was then calculated using

$$f_N = (R_g - R_f)/(R_0 - R_f) \qquad (3)$$

where R was the ratio of absorbance at 257 and 287.5 nm ($A_{257}/A_{287.5}$) and where R_f, R_0, and R_g were the absorbance ratios of the denatured enzyme, native enzyme, and enzyme equilibrated in GdnHCl, respectively. The equilibrium constant (K_D) and the free energy of denaturation (ΔG_D) were calculated for the transition using

$$K_D = (1 - f_N)/f_N \qquad (4)$$

$$\Delta G_D = -RT \ln K_D \qquad (5)$$

where $R = 8.314$ kJ mol^{-1} K^{-1} and $T = 298$ K. Regression of ΔG_D values on the corresponding GdnHCl concentrations yielded a straight line defined by

$$\Delta G_D = \Delta G_D{}^\circ + m[\mathrm{D}] \qquad (6)$$

where [D] was the concentration of GdnHCl and m measured the dependence of ΔG_D on [D]. Linear extrapolation to zero concentration denaturant yielded an estimate of the free energy of denaturation under standard conditions ($\Delta G_D{}^\circ$).

The thermodynamic reversibility of proteinase denaturation was evaluated by equilibrating samples in 4.0 M GdnHCl (pH 5.5) for 4 h at 4 °C followed by exhaustive dialysis against 20 mM citrate–phosphate buffer (pH 2.9, 4.1, 5.0, and 6.3); proteolytic activity was subsequently assayed. Under no condition was activity recoverable (results not shown); consequently, the $\Delta G_D{}^\circ$ values obtained from equilibrium analyses are qualified as apparent using the subscript "app", i.e., $\Delta G_D{}^\circ{}_{app}$. Irreversible denaturation of aspartyl proteinases and the valid use of equilibrium analyses under this condition have been previously discussed (Brown and Yada, 1991).

Structure. Circular dichroism (CD) spectra were measured using a Jasco J-600 spectropolarimeter (Japan Spectroscopic Co., Ltd., Tokyo) under a constant N_2 flush at 25 °C. Sample preparation, spectral measurement, and calculation of mean residual ellipticity (far-UV) and change in molar absorptivity (near-UV) were according to the procedures of Yada and Nakai (1986b). Protein concentration was approximately 0.05 and 0.005 mg/mL for near-UV and far-UV CD measurements, respectively. Spectra were corrected for the MES (20 mM, pH 5.5) buffer. Secondary structure fractions (α-helix, β-sheet, β-turn, and random) were determined using the Jasco Protein Secondary Structure Estimation program (Japan Spectroscopic Co.) based on the method of Chang et al. (1978).

Statistical Analysis. Data were analyzed using ANOVA and Duncan's new multiple range test (SAS Institute Inc., 1985).

Table I. Effect of Reaction Conditions on the Degree of Carboxyl Modification, Enzyme Activity, and Relative Electrophoretic Mobility (REM) of *M. miehei* Proteinase[a]

[EDC],[b] mM	[GME],[c] mM	no. of COOH groups modified	PA,[d] units/mg	MC,[e] units/mg	REM value
0	0	0.0	355[c]	1351[f]	1.00
0	50	0.0	352[c]	1357[f]	1.00
20	0	0.0	323[b]	986[e]	0.91
10	25	3.0	284[a]	621[d]	0.77
10	50	5.1	280[a]	157[b]	0.64
20	25	4.5	280[a]	284[c]	0.70
20	50	7.2	273[a]	68[a]	0.45
20	100	9.6	271[a]	54[a]	0.42
20	200	11.4	266[a]	54[a]	0.39

[a] Modifications were conducted at pH 5.5 and 25 °C for 120 min. Values are averages of six determinations. Values in each column with the same superscript are not significantly ($P > 0.05$) different. [b] 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide. [c] Glycine methyl ester. [d] Proteolytic activity; determined using acid-denatured hemoglobin. [e] Milk-clotting activity; determined using Berridge substrate.

RESULTS AND DISCUSSION

Effect of Modification Conditions on the Degree of Modification and Proteinase Function. Similar to the work of Ma and Nakai (1980) on porcine pepsin, the effects of modification conditions on the degree of nucleophile incorporation and MMP function were investigated, using glycine methyl ester as the model nucleophile. Nucleophile incorporation occurred only when EDC and nucleophile were used in combination; the number of glycine residues incorporated increased with increased concentrations of both EDC and nucleophile (Table I). Results indicated, however, that the concentration of EDC was less critical in effecting more extensive modification; i.e., a 2-fold increase in the concentration of EDC with constant nucleophile concentration resulted in fewer modified carboxyl groups than when the EDC concentration was held constant and the concentration of nucleophile was doubled (Table I). Regardless of the concentrations of the reactants, maximum incorporation of glycine residues occurred within 120 min (results not shown).

MMP incubated with EDC in the absence of methyl ester displayed decreased ($P \leq 0.05$) proteolytic and milk-clotting activity and reduced electrophoretic mobility, i.e., decreased negative charge (Table I). In a reaction mixture containing carbodiimide and protein, carbodiimide reacts readily with carboxylic acid to yield *O*-acylisourea (Hoare and Koshland, 1967). This product may undergo acyl transfer to available nucleophile (other than water) to form the modified carboxylic acid, react with water to regenerate the carboxylic acid, or rearrange to the less labile, positively charged *N*-acylurea (Hoare and Koshland, 1967; Carraway and Koshland, 1972; Timkovich, 1977). Consequently, the reduced electrophoretic mobility of EDC-modified MMP may be attributed to the attachment of positively charged EDC molecules. Alteration of the density/distribution of negative charge may have been sufficient to change protein conformation, manifested as reduced enzyme activity.

Modification of MMP using increased concentrations of EDC and nucleophile generally resulted in progressively greater reductions in proteolytic and milk-clotting activity (Table I). No further ($P > 0.05$) decrease in activity was observed with 20 mM EDC and glycine methyl ester in excess of 50 mM; these concentrations of reactants were used for all subsequent modifications.

Effect of Modification with Different Nucleophiles on Proteinase Function. A significant ($P \leq 0.05$) reduction in proteolytic and milk-clotting activity occurred following carboxyl modification by all nucleophiles (i.e., methyl esters); the extent of nucleophile incorporation and the relative decrease in proteolytic and milk-clotting activity and MC/PA were methyl ester dependent (Table II). In addition, carboxyl-modified MMP showed reduced electrophoretic mobility compared to native enzyme, indicating their decreased negative charge (Table II).

Table II. Effect of Different Nucleophiles on the Degree of Carboxyl Modification, Enzyme Activity, and Relative Electrophoretic Mobility (REM) of *M. miehei* Proteinase[a]

nucleophile[b]	no. of COOH groups modified	PA,[c] units/mg	MC,[d] units/mg	MC/PA	REM value
native	0.0	355[d]	1351[e]	3.80	1.00
GME	7.2	273[b]	68[a]	0.25	0.45
LME	11.2	309[c]	108[c]	0.35	0.74
AME	12.5	270[b]	81[b]	0.30	0.51
TME	5.1	227[a]	121[d]	0.53	0.66

[a] Modifications were conducted at pH 5.5 and 25 °C for 120 min using 20 mM EDC and 50 mM of each of the different nucleophiles. Values are averages of six determinations. Values in each column with the same superscript are not significantly ($P > 0.05$) different. [b] GME, glycine methyl ester; LME, leucine methyl ester; AME, arginine methyl ester; TME, tryptophan methyl ester. [c] Proteolytic activity; determined using acid-denatured hemoglobin. [d] Milk-clotting activity; determined using Berridge substrate.

Catalysis by aspartyl proteinases depends on several factors including the positions and ionization states of the two active site aspartates and, possibly, induced strain and/or conformational changes (Sali et al., 1989). Carboxyl modification of active site aspartates in MMP was unlikely given the continued high, albeit significantly ($P \leq 0.05$) reduced relative to native enzyme, proteolytic activity of the MMP derivatives (Table II). Continued milk-clotting activity by carboxyl-modified MMP (Table II) also argues against modification of active site aspartates. Note that greater reduction in milk-clotting activity compared to proteolytic activity may be related to the size difference between the two macromolecular substrates used to determine activity.

Pepstatin A inhibition studies also provided evidence that active site aspartates were not modified under the specified reaction conditions. Native enzyme displayed less than 1% of initial proteolytic activity after 30 min of incubation with pepstatin A, while carboxyl-modified MMP displayed between 4 and 8% of initial activity (results not shown). Incubation for 60 min resulted in complete inactivation of all species. Pepstatin A is thought to bind a portion of the substrate-binding and/or active site of aspartyl proteinases (Takahashi and Chang, 1976). Therefore, since carboxyl-modified MMP remained susceptible to inhibition by pepstatin, i.e., activity was inhibited by between 92 and 96%, it appeared that substrate-binding/active site residues were not modified. Modification of carboxyl groups in proximity to these sites resulting in steric restriction of inhibitor may account for the lower degree of inhibition of carboxyl-modified MMP when incubated for only 30 min.

On the basis of the above, reduced enzyme activity of carboxyl-modified MMP may, therefore, be attributed to (i) conformational change(s) resulting from decreased negative charge, (ii) steric restriction of substrate from substrate-binding and/or active site aspartate residues by nucleophile groups attached to catalytically nonessential residues, or (iii) a combination of (i) and (ii).

Modification of carboxyl groups in MMP caused a shift in the pH–activity profile of the enzyme. Modification with glycine and arginine methyl esters shifted the pH–activity optimum from pH 5.0 (native MMP) to pH 3.0, while MMP modified with methyl esters of tryptophan

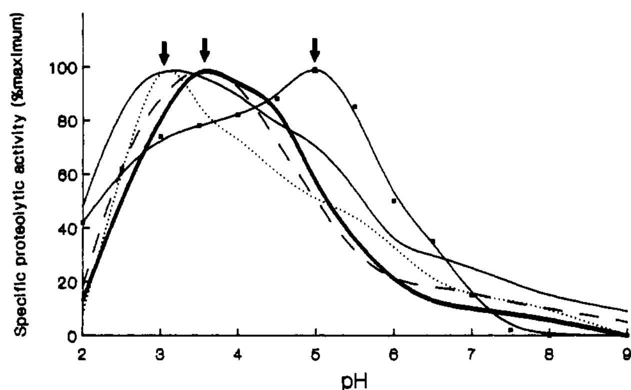

Figure 1. pH–activity profiles of native and carboxyl-modified *M. miehei* aspartyl proteinase. The pH of maximum proteolytic activity is indicated by the arrows and after each amino acid methyl ester. (■) Native, 5.0; (···) glycine, 3.0; (– –) leucine, 3.5; (—) arginine, 3.0; (━) tryptophan, 3.5. Enzyme samples were modified at pH 5.5 and 25 °C for 120 min using 20 mM EDC and 50 mM of each of the amino acid methyl esters.

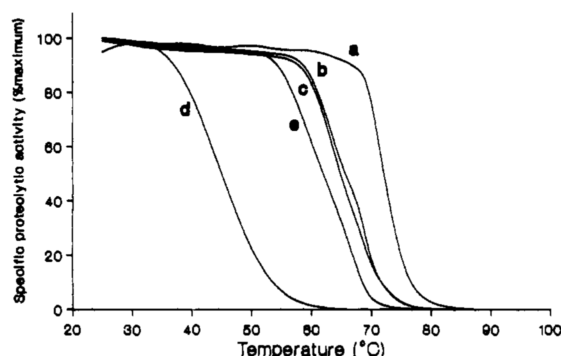

Figure 2. Temperature–activity profiles of (a) native and carboxyl-modified *M. miehei* aspartyl proteinase. Nucleophiles used were (b) glycine methyl ester, (c) leucine methyl ester, (d) arginine methyl ester, and (e) tryptophan methyl ester. Enzyme samples were modified at pH 5.5 and 25 °C for 120 min using 20 mM EDC and 50 mM of each of the amino acid methyl esters.

and leucine showed optimal activity at pH 3.5 (Figure 1). These results were consistent with the observed reductions of electrophoretic mobility and further suggested that a change in the density/distribution of negative charge occurred as a result of carboxyl modification. It was noteworthy that those amino acid methyl esters that produced the greatest reduction in electrophoretic mobility also caused the largest shift in pH–activity optimum, e.g., glycine and arginine. There was also a general trend toward increased pH–stability following modification with each of the amino acid methyl esters, i.e., modified MMP remained active to pH 9.0, whereas native enzyme showed no activity above pH 8.0 (Figure 1). Enhanced pH–stability was also observed following carboxyl modification of porcine pepsin (Ma and Nakai, 1980).

Proteolytic activity of native MMP was unaffected ($P > 0.05$) by preincubation of the enzyme at temperatures ranging from 25 to 70 °C; incremental temperature increases above 70 °C resulted in progressively greater inactivation (Figure 2). Similar trends were observed for carboxyl-modified MMP; however, the temperature range over which proteolytic activity remained unchanged ($P > 0.05$) was more narrow (Figure 2). Also, in comparison to native enzyme, the temperature range over which proteolytic activity was detectable was decreased for all of the carboxyl-modified MMP derivatives (Figure 2).

Quantitative assessment of thermostability using DSC showed that only arginine and tryptophan methyl ester-modified MMP displayed significantly ($P \leq 0.05$) lower

Table III. Effect of Modification with Different Nucleophiles on the Stability of *M. miehei* Proteinase

nucleophile	stability parameter[a]			
	ΔH_D,[b] kJ/mol	T_D,[c] °C	E_a,[d] kJ/mol	$\Delta G_D°_{app}$,[e] kJ/mol
native	90.4[c]	79.6[c]	502[e]	54.4[d]
GME	90.1[c]	79.6[c]	489[d]	45.1[c]
LME	88.7[c]	78.9[c]	472[c]	42.7[c]
AME	66.2[a]	75.3[a]	252[a]	19.3[a]
TME	75.3[b]	76.9[b]	345[b]	26.2[b]

[a] Values are averages of six determinations. Values in each column with the same superscript are not significantly ($P > 0.05$) different. [b] Enthalpy of denaturation obtained from differential scanning calorimetry (DSC) thermograms. [c] Peak denaturation temperature obtained from DSC thermograms. [d] Activation energy of denaturation obtained from DSC thermograms. [e] Conformational stability determined from transition curve analysis as described under Materials and Methods.

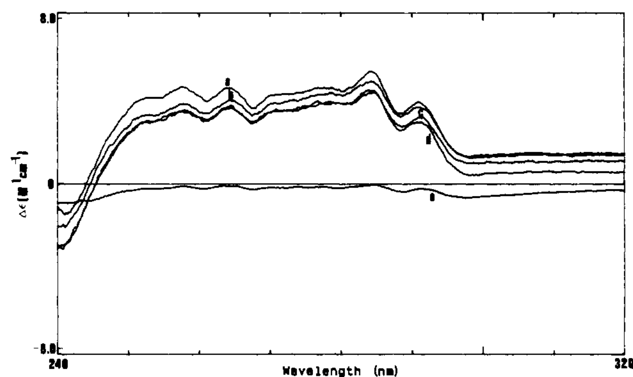

Figure 3. Near-UV CD spectra of (a) native *M. miehei* aspartyl proteinase and the same enzyme following carboxyl modification with methyl esters of (b) glycine, (c) leucine, (d) arginine, and (e) tryptophan. Modification conditions were the same as in Figure 1.

thermostability and peak denaturation temperature compared to native MMP (Table III). In contrast, the kinetic and thermodynamic parameters E_a of denaturation and $\Delta G_D°_{app}$, respectively, were significantly ($P \leq 0.05$) lower, relative to native enzyme, for all carboxyl-modified MMP samples (Table III). Altered conformational stability, i.e., $\Delta G_D°_{app}$, or thermodynamics of denaturation have similarly been reported to result from chemical modification of β-lactoglobulin, cytochrome *c*, and ribonuclease (Hollecker and Creighton, 1982). Regardless of the index of stability used, arginine and tryptophan methyl ester-modified MMP showed the lowest stability. This finding appeared, however, not to be related to the absolute number of modified carboxyl groups since no relationship between the number of modified residues and the stability parameters was observed. That is, little or no change in these parameters was observed in MMP in which approximately 7 and 11 carboxyl residues were modified using glycine and leucine methyl esters, respectively, while substantial changes were shown after modification of approximately 12 and 5 carboxyl groups using methyl esters of arginine and tryptophan, respectively.

Effect of Modification on Proteinase Structure. Carboxyl-modified MMP, and tryptophan methyl ester-modified MMP in particular, yielded near-UV CD spectra with lower intensity compared to native enzyme (Figure 3). In addition, the fine structure of the aromatic amino acids phenylalanine (257 and 264 nm), tyrosine (276 nm), and tryptophan (285 and 292 nm), apparent in the spectrum of native MMP and glycine-, leucine-, and arginine-modified MMP, was not apparent in the spectrum of tryptophan-modified MMP (Figure 3). The intensity

of near-UV CD spectra reflects protein tertiary structure (Strickland, 1974) and is influenced largely by protein rigidity, the interaction of aromatic rings with their immediate environment, and the number of constituent aromatic residues. In studies with model compounds, increased mobility of aromatic side chains decreased the intensity of CD spectra (Strickland, 1974). Carboxyl modification may have perturbed proteinase structure, thereby allowing greater mobility of constituent aromatic residues. Reduced enzyme activity and decreased susceptibility to pepstatin inhibition, together with near-UV CD spectral analyses, support the possibility of altered, i.e., perturbed, proteinase structure. The loss of fine structure in the CD spectrum of tryptophan-modified MMP may have also resulted from increased mobility of aromatic residues due to the presence of greater number of these residues (i.e., five tryptophans were incorporated). Indeed, proteins containing large numbers of aromatic amino acids may not have large CD bands as a result of cancellations by positive and negative contributions (Strickland, 1974). Changes in the intensity of near-UV CD spectra of modified MMP were associated with changes in physicochemical properties, as previously discussed. No apparent relationships were observed between the type of nucleophile used, the extent of carboxyl modification, the physicochemical properties, or the degree of alteration of tertiary structure.

In contrast to near-UV CD spectra, far-UV CD spectra (a reflection of protein secondary structure; Johnson, 1988) of carboxyl-modified MMP did not differ from that of native enzyme; i.e., there were no significant ($P > 0.05$) differences between the proportions of secondary structure fractions. On average, native and carboxyl-modified MMP contained less than 0.1% α-helix, 60% β-sheet, 15% β-turn, and 25% random (results not shown). Yada and Nakai (1986b) similarly observed that loss of enzyme activity, together with altered tertiary structure, was not coincidental with changes in secondary structure.

The successful use of a (aspartyl) proteinase to enzymatically coagulate milk for cheese-making requires that it have comparable milk-clotting activity and MC/PA and be no more thermostable than the current industry standard chymosin. This study endeavored to test the hypothesis that the MC/PA of aspartyl proteinases was, in part, inversely related to negative charge. Results obtained indicated, however, that reduction of negative charge in MMP by chemical modification of ionizable carboxyl groups (e.g., γ and δ carboxyl groups of glutamic and aspartic acids, respectively) decreased proteolytic and milk-clotting activity, with the latter of these being affected more. Decreased stability was also a consequence of carboxyl modification but only by certain nucleophiles. This finding suggested that the properties of the different nucleophiles, e.g., charge, size, hydrophobicity, possibly in conjunction with charge alteration by them, were critical in effecting proteinase destabilization. Evidence from CD analyses suggested that the activity reductions and decreased stability were the result of modification-induced disruption of proteinase tertiary structure.

Finally, the locations of the carboxyl groups involved in the above-described modifications are not known from the results obtained here. Consequently, variable alteration of the structure–function and stability of MMP by the different nucleophiles may be a reflection of their differing affinities for carboxyl groups in different locations of the enzyme; identification of these carboxyl groups awaits more detailed structural analysis.

CONCLUSIONS

Reduced thermostability was demonstrated for arginine and tryptophan methyl ester-modified MMP; however, the drastically reduced MC/PA of these MMP derivatives precludes the use of this modification strategy for improving the cheese-making potential of MMP. Alteration of other parameters, e.g., hydrophobicity, possibly in concert with charge reduction, and their effects on MC/PA are currently being investigated.

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