Formation and Analysis of [(Phenylethyl)amino]alanine in Food Proteins

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[(Phenylethyl)amino]alanine (PEAA), lysinoalanine (LAL), and all other amino acids can be assayed on an amino acid analyzer. PEAA appears as a single, well-resolved peak at 97 min, eluting after arginine, as determined with (a) synthetic PEAA added to a standard mixture of amino acids or cohydrolyzed with casein and (b) protein-bound PEAA in casein and soybean proteins. The elution time of PEAA can be reduced to 43 min by modification of the chromatographic conditions. The calculated recovery of PEAA from the cohydrolysate was 100%. The area of the PEAA peaks on amino acid chromatograms was proportional to concentration. The lower limit of sensitivity of analysis is estimated to be about 1 nmol of PEAA on the column. Related experiments indicate that the extent of formation of PEAA in casein, acetylated casein, soybean protein, and acetylated soybean protein is consistent with a postulated mechanism for its formation from phenylethylamine and dehydroalanine and that LAL and PEAA formation are competitive reactions.

INTRODUCTION

The biogenic amine phenylethylamine interacts with dehydroalanine residues of food proteins to form [(phenylethyl)amino]alanine (PEAA) side chains, analogous to the way nucleophilic addition reactions of the ϵ-NH_2 group of lysine form lysinoalanine (LAL) residues (Jones et al., 1981; Tucker and Jones, 1983; Friedman et al., 1984a). Jones et al. used a ^{14}C-labeled amine and detection of radioactive zones on electrophoretograms of acid-hydrolyzed proteins to detect the presence of PEAA in proteins. Because this method may be too expensive and time consuming, alternate possibilities need to be explored for estimating PEAA.

Figure 1 depicts a postulated mechanism for the simultaneous formation of LAL and PEAA in a protein. Hydroxide ions induce β-elimination reactions in cystine and serine to form dehydroalanine side chains. The double bond of dehydroalanine can then interact with ϵ-NH_2 groups of lysine side chains to produce LAL cross-links (Patchornik and Sokolovsky, 1964; Friedman et al., 1984a) and with primary NH_2 groups of added phenylethylamine to form PEAA side chains (Jones et al., 1981). Lysinoalanine was previously shown to elute on amino acid chromatograms as a single, well-resolved peak before histidine under the conditions cited in the Experimental Section (Friedman et al., 1984a). The main objectives of the present study were to find out whether PEAA, LAL, and other amino acids could be determined by ion-exchange chromatography in a single assay using standard amino acid analysis techniques and to assess the factors that influence the competitive formation of LAL and PEAA in casein and soybean proteins.

EXPERIMENTAL SECTION

Materials. [(Phenylethyl)amino]alanine monohydrochloride was synthesized from phenylethylamine and *N*-acetyldehydroalanine as described by Jones et al. (1981) for the *p*-toluenesulfonate salt. The compound was purified by recrystallization from 95% ethanol. Its nuclear magnetic resonance (NMR) spectrum in trifluoracetic acid was consistent with the assigned structure. The standard amino acid calibration mixture was obtained from Beckman Laboratories, Palo Alto, CA. Casein was obtained from Nutritional Biochemicals Corp., Cleveland, OH. Soybean protein (Promine-D) was a gift of Central Soya, Ft. Wayne, IN.

Acetylated Proteins. One kilogram of protein (casein or soybean protein) was suspended in 2.5 L of saturated sodium acetate solution and 2.5 L of deionized water. A smooth slurry was prepared in a Waring blender. The pH of the casein slurry was 6.3 and of the soybean protein slurry, 7.2. The material was cooled at 0 °C in an ice water–salt bath. Acetic anhydride (900 mL) then was added dropwise over a period of about 2 h while the slurry was being stirred. Stirring was continued for another 1 h before the sample was placed in the cold room for 16 h. The material was then dialyzed against dilute (0.01 N) sodium hydroxide to neutralize the pH, dialyzed against distilled water, and lyophilized.

Extent of Acetylation. The extent of acetylation of protein amino groups was estimated by an improved manual ninhydrin procedure (Friedman et al., 1984b): for casein, $As_{570nm} = 0.830$; for acetylated casein, As = 0.237; % acetylated casein = $(0.830 - 0.237/0.830) \times 100 = 71\%$. An analogous experiment showed that soybean protein was 80% acetylated.

Treatments. Nitrogen was bubbled into protein solutions (1 g of protein in 100 mL of 0.1 N NaOH and 1 mL of phenylethylamine) for 3 min in 250-mL Erlenmeyer flasks with ground joints. The flasks were then covered with greased stopcocks that were tightly secured with rubber bands. Next, the flasks were heated in a 75 °C water bath for 3 h. Control experiments without phenylethylamine were run concurrently. The samples were then dialyzed against 0.01 N acetic acid for 1 day and against distilled water for 2 days more. Finally, the samples in the dialysis tubes were lyophilized.

For the pH study, acetylated casein plus phenylethylamine were dissolved in deionized–distilled water, and the pH was adjusted to the desired range with either 4 N NaOH or 6 N HCl before the samples were placed for 3 h in a 75 °C water bath.

Amino Acid Analysis. A weighed sample of 5 mg of protein was hydrolyzed in 10 mL of 6 N HCl in a commercial hydrolysis tube. The tube was evacuated with the aid of a vacuum pump, placed in an acetone–dry ice bath, again evacuated, and flushed with nitrogen before being placed in an oven at 110 °C for 24 h. Next, the hydrolysate was cooled, filtered through a disk funnel, and evaporated to dryness at 40 °C with the aid of an aspirator. The

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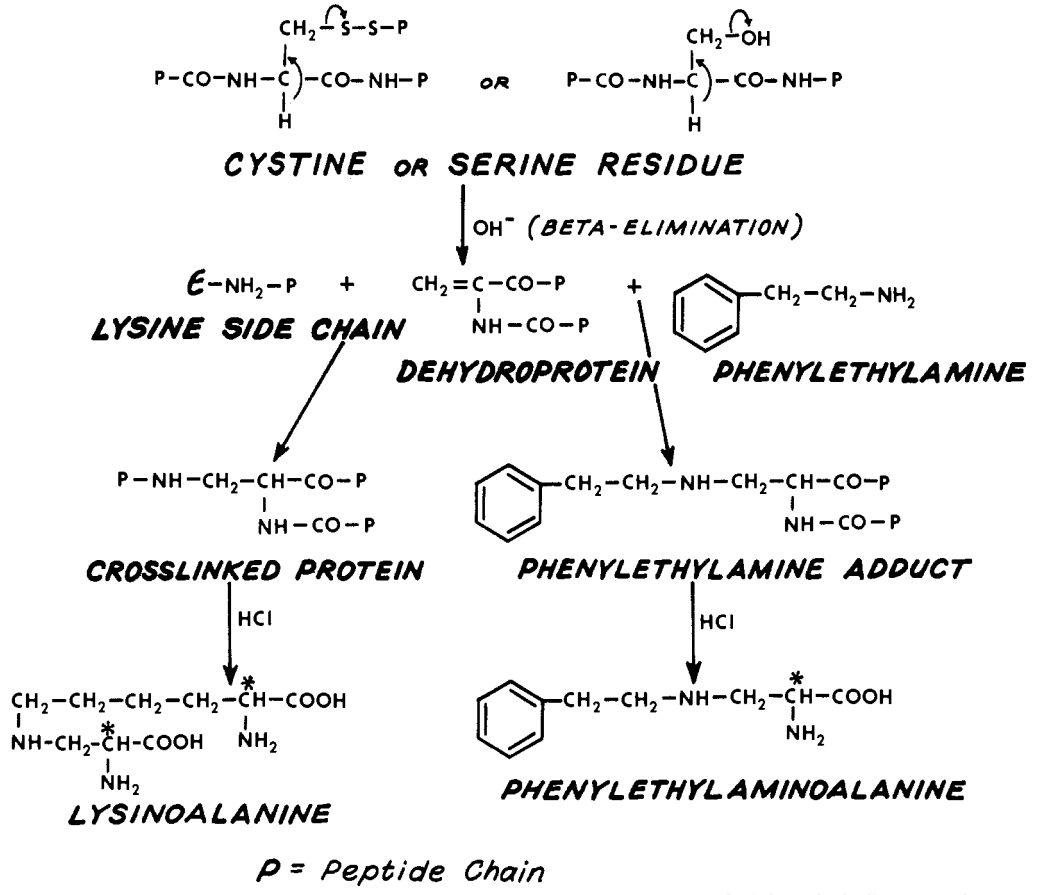

Figure 1. Transformation of cystine and serine residues in a protein to lysinoalanine and [(phenylethyl)amino]alanine. Newly created asymmetric centers are indicated by asterisks.

residue was suspended in water and again evaporated to dryness. Amino acid analysis of an aliquot of soluble hydrolysate was carried out on a Durrum amino acid analyzer, Model D-500, under the following conditions: buffer pH 3.25, 4.25, and 7.90; photometer 440 and 590 nm; column 40 cm × 1.75 mm i.d.; column temperature 51.6 °C; analysis time 110 min. Norleucine was added as an internal standard.

For the spiking experiments, a solution of PEAA (4 μg in 20 μL of 0.1 N HCl) was added to 20 μL of the standard amino acid mixture and applied to the column.

For the cohydrolysis experiments, 0.4 mg of synthetic PEAA was added to 5 mg of casein or to 5 mg of starch in triplicate hydrolysis runs.

Experiments were also carried out with one pH 7.9 buffer column, a temperature of 66.4 °C, and an analysis time of 50 min.

The reproducibilites for separate assays are estimated to be ±3% of better (Friedman et al., 1979).

Gas Chromatographic Analysis. PEAA was analyzed as the *N*-pentafluoropropyl isopropyl ester on a Chirasyl-VAL III capillary column (Applied Science, Deerfield, IL) as described by Tovar and Schwass (1983). Norleucine was used as an internal standard.

RESULTS AND DISCUSSION

Analysis of [(Phenylethyl)amino]alanine by Ion-Exchange Chromatography. Since single-column amino acid analyzers are now widely used to measure amino acids, including lysinoalanine, in food proteins, we describe a procedure for analyzing PEAA and LAL along with all other amino acids in a single determination. Application of this procedure to casein and soybean proteins and the corresponding acetylated derivatives permits conclusions about the competition between LAL and PEAA formation.

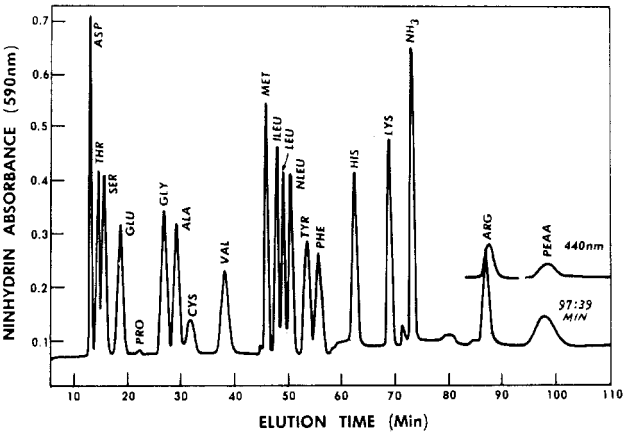

Figure 2. Elution of [(phenylethyl)amino]alanine (PEAA) added to a standard amino acid calibration mixture. A solution of PEAA (20 μL of 0.747 μM/mL in 0.1 N HCl) plus 20 μL of the calibration mixture was applied to the column and eluted with the three buffers described in the Experimental Section.

An ideal method for analysis of an amino acid with an amino acid analyzer should meet the following requirements: (a) the amino acid should be eluted in a convenient position as a separate peak; (b) the intensity of the peak should vary linearly with concentration; (c) the amino acid should be stable to the strong-acid conditions used for protein hydrolysis. The following experiments tested the applicability of these requirements to PEAA: First, synthetic PEAA was added to a standard amino acid calibration mixture. The regular analysis time was extended from 90 to 110 min with no other modification. Figure 2 shows that PEAA eluted as a well-resolved peak at about 97 min, approximately 10 min after arginine. Cohydrolysis

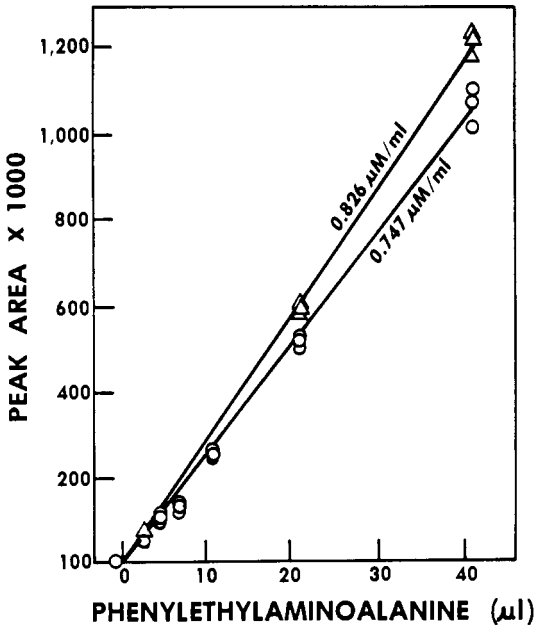

Figure 3. Plots of PEAA concentration vs. integrated areas of peaks on amino acid analysis chromatograms.

of synthetic PEAA with casein or hydrolysis of protein-bound PEAA in casein and soybean proteins did not alter its elution position.

To test the relationship between concentration of PEAA and peak areas on chromatograms, solutions of PEAA containing 0.747 or 0.826 μM/mL in 0.1 N HCl were applied to the amino acid analyzer in different amounts from 2 to 40 μL, equivalent to 1.5–33 nmol of PEAA. Figure 3 shows that plots of concentration vs. area on the chromatograms were linear in both cases.

The recovery of PEAA cohydrolyzed with casein was quantitative (from triplicate determinations). Additional studies showed that (a) the recovery of PEAA cohydrolyzed with starch was 64.6 ± 2%; (b) the ninhydrin color factor of PEAA on an amino acid analyzer was 0.60 (leucine = 1.0); and (c) the minimum amount of PEAA that can be estimated on the ion-exchange column was about 1 nmol.

The effects of varying buffer pH and column temperature showed that the elution time of PEAA can be reduced to about 43 min by (a) using a single pH 7.9 citrate buffer as eluant instead of the three-buffer system described in the Experimental Section and (b) raising the column temperature from 51.6 to 66.4 °C. Although this modification causes some of the amino acid peaks to coalesce on the chromatograms (Figure 4), it should nevertheless be useful for rapid assays of PEAA, but not LAL or all of the other amino acids.

Attempts to resolve the peak associated with phenylethylamine, the precursor of PEAA, were not successful. This result is in contrast to the previously reported assay for the related amine histamine under conditions similar to those used for PEAA (Friedman and Noma, 1981). The results of this and the previous study suggest that both histamine, which eluted at 120 min, and PEAA, which eluted at 97 min under the cited conditions, can be analyzed simultaneously by ion-exchange chromatography.

In related studies, it was shown that 2-(mercaptoethyl)pyridine interacts rapidly with dehydroalanine residues in alkali-treated casein and acetylated casein to form 2-*S*-(pyridylethyl)cysteine side chains (Masri and Friedman, 1982). Since the (pyridylethyl)cysteine content of proteins can also be estimated by the described amino acid analysis technique (Friedman et al., 1970, 1979, 1980), it

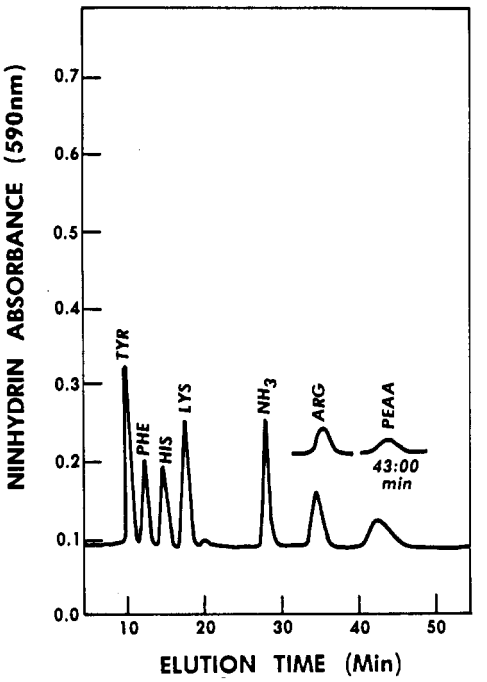

Figure 4. Elution position of PEAA under modified ion-exchange chromatographic conditions. A solution of PEAA (10 μL of 0.826 μM/mL) plus standard amino acid calibration mixture (10 μL) was applied to the column and eluted with pH 7.9 citrate buffer only. The acidic and neutral amino acids appear together before Tyr.

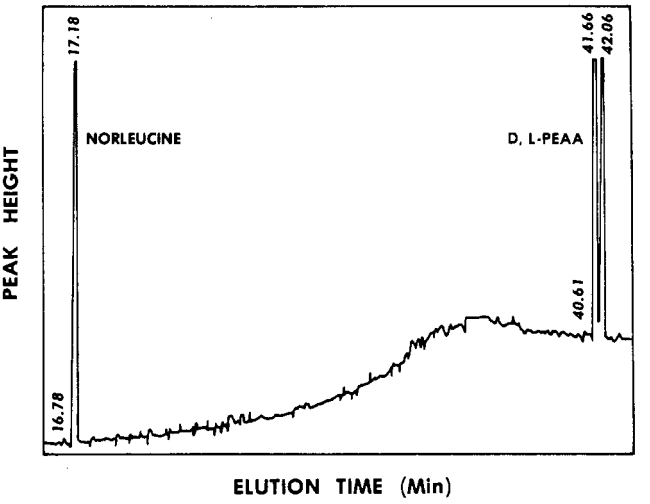

Figure 5. Gas chromatographic resolution of a volatile derivative of synthetic PEAA on a chiral column. Note two peaks at 41.66 and 42.06 min, presumably associated with the D and L enantiomers and the norleucine peak added as an internal standard at 17.18 min.

was suggested that this transformation may be useful as a chemical basis for an analytical method for dehydroalanine residues in peptides and proteins.

The SH group of cysteine (Strumeyer et al., 1963; Gross, 1977; Asquith and Otterburn, 1977; Friedman et al., 1977) and of benzyl mercaptan (Eiger and Greenstein, 1948; Gavaret et al., 1980) also adds to the double bond of dehydroalanine residues in proteins to form lanthionine and benzylcysteine, respectively. These two amino acids can also be determined on an amino acid analyzer.

Isomeric Composition of [(Phenylethyl)amino]alanine. Since a new asymmetric center is created during the formation of PEAA from phenylethylamine and dehydroalanine (Figure 1), synthetic PEAA should consist of an equimolar mixture of D and L enantiomers. Preliminary

Table I. Effect of Temperature on the Formation of Lysinoalanine and [(Phenylethyl)amino]alanine in Acetylated Casein[a,b]

temp, °C	lysine	lysinoalanine	[(phenylethyl)amino]alanine
25	47.2	0.0	0.0
45	45.1	1.2	2.6
65	43.7	2.9	13.9
75	42.5	4.3	15.8
85	47.2	3.7	12.1
95	45.8	2.8	7.8
untreated casein	50.9	0.0	0.0
untreated acetylated casein	51.4	0.0	0.0

[a] Acetylated casein plus phenylethylamine were heated at the indicated temperatures for 3 h. Data for untreated controls are included for comparison. [b] Listed values are in units of mmol/100 g.

Table II. Effect of Time of Treatment on the Formation of Lysinoalanine and [(Phenylethyl)amino]alanine in Acetylated Casein[a,b]

time, min	lysine	lysinoalanine	[(phenylethyl)amino]alanine
10	47.0	0.8	0.0
30	46.6	2.1	8.1
60	46.6	2.8	12.0
120	45.9	3.3	16.2
180	42.5	4.3	15.8

[a] Acetylated casein plus phenylethylamine in 0.1 N NaOH were heated at 75 °C for the indicated time periods. [b] Listed values are in units of mmol/100 g.

Table III. Effect of pH on the Formation of Lysinoalanine and [(Phenylethyl)amino]alanine in Acetylated Casein[a,b]

pH	lysine	lysinoalanine	[(phenylethyl)amino]alanine
5	48.0	0.0	0.0
6	47.1	0.0	0.0
7	45.4	0.0	0.0
8	45.5	0.0	0.0
9	46.8	0.0	0.0
10	46.0	0.0	0.0
11	50.7	0.0	0.0
12	45.9	0.7	2.2

[a] Acetylated casein plus phenylethylamine was brought to the desired pH with NaOH or HCl and then heated at 75 °C for 3 h. [b] Listed values are in units of mmol/100 g.

results in Figure 5 show that this is indeed the case since synthetic PEAA eluted as two peaks of equal height on a chiral gas chromatographic column that separates optical enantiomers of amino acids (Tovar and Schwass, 1983). The compound is therefore correctly named 3-[*N*-(β-phenylethyl)amino]-DL-alanine. The general applicability of the gas chromatographic procedure to the analysis of PEAA in proteins awaits further study.

Mechanism of Formation of [(Phenylethyl)-amino]alanine. To define the factors that may influence PEAA formation, acetylated casein was exposed to phenylethlamine at different temperatures (Table I), for different periods (Table II) in 0.1 N NaOH, and in aqueous media in the pH range 5–12 (Table III). The results show that PEAA formation increased with temperature up to 75 °C and then decreased. It also increased with time, up to 2 h and then decreased. Table III shows that PEAA is not produced at all in the pH range 5–10 but starts forming when the medium reaches a pH between 11 and 12.

The reason why PEAA formation as function of temperature goes through a maximum (Table I) is not immediately apparent. The following are some possible explanations for this effect: First, either PEAA or its dehydroalanine precursor may be unstable at the higher temperatures, i.e. they are degraded at a faster rate than they are formed. Second, hydrolytic reactions, which are more pronounced at the higher temperatures, might cleave small peptide fragments rich in serine residues (the dehydroalanine precursor) that are dialyzed away during the workup of the reaction mixture (cf. Friedman and Liardon, 1985). Third, the nucleophilic addition reaction leading to PEAA formation (Figure 1) may be partly reversible at the higher temperatures. Additional studies are needed to clarify these aspects of PEAA formation.

Additional experiments were carried out to further clarify the postulated mechanism of PEAA formation in food proteins. As Figure 1 indicates, the same intermediate, dehydroalanine, is postulated to compete for the primary NH_2 groups of lysine and phenylethylamine. Consequently, blocking of the ε-NH_2 groups of lysine by acetylation should minimize or prevent LAL formation, leaving a greater fraction of dehydroalanine residues to interact with the NH_2 groups from phenylethylamine to form PEAA. Results in Tables IV and V show that these expectations were indeed realized for alkali-treated casein and soybean proteins and the corresponding partly acetylated derivatives.

The data in Table IV show that subjecting casein to the cited alkaline conditions for 1 or 3 h produces, respectively, 3.20 and 4.60 g/16 g of N of LAL. Analogous treatment of casein in the presence of phenylethylamine results in a decrease in LAL content to 1.94 and 2.60 g/16 g of N, respectively. This decrease is accompanied by the formation of PEAA (3.03 and 4.56 g/16 g of N). The data in the table also show that alkaline treatment of acetylated casein for the same time periods lowers the LAL content to 1.30 and 1.69 g/16 g of N. The corresponding treatment of acetylated casein in the presence of phenylethylamine resulted in a further decrease in LAL to 0.606 and 0.851 g/16 g of N. There was no change in PEAA for the sample treated for 1 h and an increase to 6.5 g/16 g of N for the 3-h sample.

Table IV. Effect of Alkali Treatment in the Absence and Presence of Phenylethylamine (PA) on the Thr, Ser, Lys, Arg, Lysinoalanine (LAL), and [(Phenylethyl)amino]alanine (PEAA) Content of Casein[a]

amino acid	casein, untreated	casein, treated[b]		casein + PA, treated		acetylated casein, treated		acetylated casein + PA, treated	
		1 h	3 h	1 h	3 h	1 h	3 h	1 h	3 h
Thr	4.84	3.59	3.03	3.72	3.40	3.53	2.95	3.73	3.04
Ser	6.58	4.06	3.00	4.28	2.77	3.94	2.97	4.00	2.49
Lys	9.27	6.36	5.71	7.44	7.27	7.63	7.73	8.19	7.80
Arg	4.38	3.42	2.98	3.88	3.28	3.36	2.87	3.68	3.05
LAL	0.00	3.20	4.60	1.94	2.60	1.30	1.69	0.606	0.851
PEAA	0.00	0.00	0.00	3.03	4.56	0.00	0.00	2.93	6.50

[a] Listed values are in units of g/16 g of N. [b] Conditions: 1% protein in 0.1 N NaOH at 75 °C for the indicated time periods.

Table V. Effect of Alkali Treatment in the Absence and Presence of Phenylethylamine (PA) on the Thr, Ser, Lys, Arg, Lysinoalanine (LAL), and [(Phenylethyl)amino]alanine (PEAA) Content of Soy Proteins[a,b]

amino acid	soy protein, commercial	soy protein, dialyzed control	soy protein, treated		soy protein + PA, treated		acetylated soy protein, treated		acetylated soy protein + PA, treated	
			1 h	3 h	1 h	3 h	1 h	3 h	1 h	3 h
Thr	3.79	4.07	3.23	2.71	3.50	2.90	3.21	2.67	3.41	2.86
Ser	5.41	5.52	4.49	3.72	4.46	3.39	4.49	3.65	4.60	3.30
Lys	6.42	6.23	5.25	4.91	5.69	5.54	6.31	6.33	6.50	6.15
Arg	8.00	8.08	6.81	6.00	7.47	6.24	6.94	5.83	7.43	5.99
LAL	0.00	0.00	7.76	2.84	1.14	1.43	0.446	0.698	0.216	0.543
PEAA	0.00	0.00	0.00	0.00	1.87	1.44	0.00	0.00	2.82	2.35

[a] Listed values are in units of g/16 g of N. [b] Conditions: 1% protein in 0.1 N NaOH at 75 °C for the indicated time periods.

A parallel study with soybean proteins and soybean proteins in which the amino groups were 80% acetylated (as estimated by the ninhydrin reaction) shows that acetylation appears responsible for a similar decrease in LAL and increase in PEAA content (Table V). Unlike the described trends with casein, however, the limited data with soybean proteins show that although the extent of LAL formation increases with time, as expected, PEAA formation in both soy proteins and acetylated soy proteins does not. Additional studies are needed to better define the factors that influence PEAA generation in structurally different food proteins.

Examination of the amino acid profiles of untreated and alkali-treated proteins (Tables IV and V) revealed that LAL and PEAA formation was accompanied by a time-dependent decrease in the content of the following amino acids: Thr, Ser, Lys, Arg. Thus, in terms of g/16 g of N, the value of 4.84 for Thr in untreated casein decreased to 3.59 and 3.03 for the casein samples treated for 1 h and 3 h, respectively (Table IV). The corresponding values for Ser are 6.58, 4.06, and 3.00; for Lys, 9.27, 6.36, and 5.71; and for Arg, 4.38, 3.42, and 2.98. Similar changes occurred with acetylated casein, except that there was less of a decrease in Lys. For the treated samples, the higher lysine content (7.63 and 7.73 g/16 g of N, respectively) for the two acetylated caseins compared to that of the unacetylated ones (6.36 and 5.71 g/16 g of N, respectively) demonstrates the protective effect of acetylation against loss of lysine (Friedman, 1978; Friedman and Masters, 1982).

Similar trends are discernible for these amino acids in treated soybean and acetylated soybean proteins (Tables IV and V). The data also show that the presence of phenylethylamine during the treatment of both proteins did not influence the susceptibilities of the labile amino acids to modification or degradation.

Since the molar ratios of the remaining amino acids to Leu remained constant during the treatments, they are assumed to be unaffected by the treatments and are not included in Tables IV and V.

These observations are in accord with the competitive pathways for the formation of LAL and PEAA shown in Figure 1. The data thus support the depicted mechanism for the formation of both LAL and PEAA. The results also imply that PEAA should form more readily in proteins with high cystine and serine and low lysine contents. Additional studies are needed to verify this prediction.

The fact that two PEAA enantiomers are produced rules out an alternate mechanism of formation of PEAA involving direct nucleophilic displacement of serine hydroxyl groups or cystine disulfide bonds by the amino group of phenylethylamine. Since such displacements do not generate a new asymmetric center, this mechanism predicts the formation of only one (L) PEAA isomer. A fraction of the L form could, however, racemize to the D form under alkaline conditions, eventually reaching equilibrium at equimolar concentrations of the two isomers (cf. Masters and Friedman, 1979; Friedman and Liardon, 1985).

In conclusion, studies with synthetic PEAA and with protein-bound PEAA in casein and soybean proteins indicate that the amino acid analysis method can be used to assay LAL, PEAA, and all other amino acids in a single determination. This method should be useful in future studies on the possible occurrence of PEAA in commercial foods and on the nutritional and toxicological significance of both free and protein-bound PEAA. In this connection, it is worth noting that both LAL and PEAA can inhibit the enzymatic activities of metalloenzymes such as carboxypeptidase A and polyphenol oxidase (Friedman et al., 1985, 1986a,b). The possible nutritional and pharmacological consequences of removal of migraine-causing phenylethylamine from some foods through PEAA formation merit further study.

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Registry No. PEAA, 80267-23-8; LAL, 18810-04-3; phenylethylamine, 64-04-0; dehydroalanine, 1948-56-7.

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Effect of Microbial Rennets on Meat Protein Fractions

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Proteases of *Mucor miehei* and *Endothia parasitica*, used commercially as microbial rennets, were incubated with isolated myofibrillar, sarcoplasmic, and insoluble connective tissue fractions of meat to evaluate their potential for meat tenderization. SDS–polyacrylamide gel electrophoresis of incubation mixtures demonstrated that *E. parasitica* proteases cleaved all myofibrillar and sarcoplasmic proteins within 24 h, as did a commercial papain preparation. In addition, only papain demonstrated effective proteolysis of the insoluble connective tissue. A stable 130 000–140 000 Da degradation product of myosin resulted from action of *M. miehei* enzymes. These results suggest that *M. miehei* proteases may prove useful in meat tenderization when limited proteolysis of myosin is desired.

INTRODUCTION

Tenderness in meat can be directly related to the structure of components of its myofibrillar and connective tissue fractions (Bailey, 1972). Approaches to increasing the perceived tenderness have utilized either enzymatic or mechanical means to alter these structures. The majority of the enzymatic efforts have utilized proteases of animal or plant origin with economic considerations favoring the use of plant proteases such as bromelain, papain, and ficin. Varying positive results have been attained by antemortem injection or spraying, dipping, or multiple needle injection of meat pieces with solutions of the plant enzymes (Flynn, 1975). Papain, often containing chymopapain, is presently being used for the tenderization of beef (Brocklehurst et al., 1981).

The major effect of papain on meats has been shown to be the proteolysis of the myofibrillar protein fraction (Kang and Rice, 1970) especially of actin and myosin (Rattrie and Regenstein, 1977). Additional reports (Miyada and Tappel, 1956; Kang and Warner, 1974) also suggest proteolysis of connective tissue components by papain; however, it has not been clearly demonstrated that this action is on the insoluble connective tissue. Nevertheless, increased tenderness is observed following papain treatment. An undesirable "mushy" mouth feel results, however, if proteolysis is too extensive.

Comminution of selected meat cuts and restructuring of the pieces into formed products is another method of improving textural qualities. This effect is exerted primarily on the connective tissue. For selected products a combination of mechanical and enzymatic approaches may produce the best results. A significant reduction in binding of comminuted meat pieces occurred with the addition of papain, however (Schnell et al., 1973). This decrease in binding was probably due to degradative action of the papain on extracted myosin, the primary component responsible for binding of meat pieces (Macfarlane et al., 1977; Ford et al., 1978; Turner et al., 1979; Siegel and Schmidt, 1979; Schmidt and Trout, 1982).

In order to identify proteases having more desirable specificities than papain toward muscle proteins, i.e. less activity on myofibrillar proteins and more activity on connective tissue components, selected microbial proteases were evaluated for activity on isolated meat fractions. Protease preparations of *Mucor miehei* and *Endothia parasitica*, commercially available and widely used by the cheese industry as microbial rennets, were selected since they demonstrate differing specific peptide bond cleavage (Whitaker, 1970; Ottesen and Rickert, 1970).

MATERIALS AND METHODS

Enzymes. Rennet preparations from *M. miehei* were obtained from Chr. Hansen's Laboratory, Inc. (*Mucor miehei*$_1$), and Pfizer Chemical Division (*Mucor miehei*$_2$) both of Milwaukee, WI. A rennet preparation from *E. parasitica* was also supplied by Pfizer Chemical Division. A commercial preparation of papain, donated by Swift Independent Packing Co., Oak Brook Terrace, IL, was used for comparison of enzyme effects. Chromatographically purified collagenase from *Clostridium histolyticum* was purchased from Sigma Chemical Co., St. Louis, MO.

Proteolytic activities were determined by a modification of the method of Arnon and Shapira (1967) using a purified α_s-casein complex substrate (gift of Dr. H. M. Farrell, Jr.). An aliquot (825 μL) of a prewarmed 1% casein solution in 0.05 M sodium acetate, pH 5.5, was added to 175 μL of buffer containing varying amounts of enzyme (5–350 μg) in the presence or absence of papain activating agents, 2 mM ethylenediaminetetraacetic acid (EDTA) and 5 mM cysteine. After 10-min incubation at 37 °C, 1.5 mL of 5% trichloroacetic acid (TCA) was added, and the mixture was

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