Evidence for Lysinomethylalanine Formation in Model Systems

Barbara Kruse and Hans Steinhart*

Threonine (Thr) gives rise to the formation of a dehydro amino acid, methyldehydroalanine (MDHA) (2-aminobut-2-enoic acid), by β-elimination in alkaline medium. MDHA can react with functional groups within protein chains, for example with the ε-amino group of lysine to form lysinomethylalanine (LMeAL), a cross-link not clearly demonstrated before now. To be able to show the presence of LMeAL in peptides, the intermediate product MDHA was prepared first in the form of a stable derivative of the (*tert*-butyloxycarbonyl)methyldehydroalanine isopropyl ester (BOC-MDHA-IPE). This was followed by the synthesis of LMeAL by the reaction of BOC-MDHA-IPE with *N*-α-acetyl-L-lysine. The formation of LMeAL in threonine-containing di- and tripeptides with added *N*-α-acetyl-L-lysine was studied at different pH values within the alkaline range.

The technological treatment of vegetable proteins serves to improve structure, remove harmful and bitter substances, and increase digestibility. The treatment with alkalis (pH 8–12) is often an unavoidable part of the processing mechanism, for example to produce isolates or concentrates of vegetable proteins. This treatment, however, can lead to a damage of the proteins by loss of some limiting essential amino acids. The use of soybean protein isolate, for instance, in beverages, meat, and bakery products will increase worldwide during the next few years, because of its effect to reduce serum cholesterol and obesity (Carroll, 1987). Knowing the cause of possible damage of important ingredients during processing may help to avoid those drawbacks by changing the process parameters.

Particularly endangered are the essential amino acids threonine (Thr), lysine (Lys), and methionine (Met), as well as the nonessential amino acids serine (Ser) and cysteine (Cys). While reactions of Lys, Met, Ser, and Cys certainly have been studied in detail, both as free amino acids as well as part of peptides and proteins (de Groot and Slump, 1969; Asquith and Otterburn, 1977; Liardon and Hurrell, 1983), only a few studies on Thr are available. Possible reactions of Thr are racemization, retro aldol cleavage, and β-elimination (Figure 1). In the β-elimination reaction, dehydroalanine (DHA) is formed from Ser and 3-methyldehydroalanine (MDHA) from Thr. Until now, the formation of MDHA has not been clearly demonstrated by means of proper experiments. DHA can react with the ε-amino group of Lys, for example, to form lysinoalanine, (LAL), well investigated by many authors (Bohak, 1964; Friedman, 1977; Gould and MacGregor, 1977). MDHA is also expected to react with the ε-amino group of Lys to form lysinomethylalanine (LMeAL). Since LAL and LMeAL possibly function as cross-linking amino acids, inevitable inter- and intramolecular interactions in the form of covalent bonds are to be expected during their formation. Consequences result from this with regard to a lower digestibility of the proteins (Finot, 1983; Maga, 1984), losses of essential amino acids, and possibly a certain toxicological relevance (Woodard and Alvarez, 1967).

The goal of this study was to investigate the β-elimination reaction of Thr residue in peptides under alkaline conditions. By means of five Thr-containing di- and tripeptides the formation of LMeAL in the presence of Lys was to be studied. It was a precondition for undertaking these studies to synthesize the MDHA and from this LMeAL in order to get reference substances for the analytical proof.

Institut für Biochemie und Lebensmittelchemie der Universität Hamburg, Grindelallee 117, D-2000 Hamburg 13, FRG.

EXPERIMENTAL SECTION

Reagents: threonine (Degussa), isopropyl alcohol, HCl gas, tetrahydrofuran, toluene, dioxane, silica gel (70–230 mesh), triethylamine, *N,N'*-carbonyldiimidazole, TLC plates silica gel GF_{254} (Merck); *N*-α-acetyl-L-lysine (Serva); NaOH, concentrated HCl, phosphorus cellulose, peptides (Serva); trifluoroacetic anhydride, dichloromethane, sodium citrate, caprylic acid, standard mixture amino acids (Sigma); amino acids (Degussa); ninhydrin (Merck).

Amino Acid Analyses. Amino acids analyses of aliquots of the hydrolysates of the reaction products from the peptide reactions and of the synthesized LMeAL and LAL were carried out on a Beckmann 119 BL amino acid analyzer under the following conditions: column diameter, 9 mm; cation-exchange resin, Beckmann M 72; flow rate, 70 mL/h; temperature program, T_1 = 49 °C, 0–26 min, T_2 = 60 °C. Buffer: pH 3.26, 0–49 min; pH 4.12, 49–75 min; pH 5.60, 75–147 min.

Gas Chromatography (GC)–Mass Spectrometry (MS). Capillary GC was carried out by using a 50-m glass capillary (0.32-mm i.d.) coated with SE 30, temperature program 100–290 °C at 4 °C/min, Hewlett-Packard 5990 A. Additionally, a Finnigan MAT 8230 gas chromatograph–mass spectrometer–data system was used for acquisition of mass spectral data: ionization voltage, 70 eV; 25-m DB-5 capillary column (0.32-mm i.d.).

^{1}H NMR and IR Spectroscopies. ^{1}H NMR spectra were recorded at 400 MHz on a Bruker WM 400 NMR spectrometer in $CDCl_3$ solution (for BOC-MDHA-IPE) and in D_2O (for LMeAL). Chemical shifts are with reference to tetramethylsilane (Me_4Si) as internal standard. Infrared spectra were obtained from a potassium chloride molded piece with a Pye Unicam SP 2000 instrument.

Preparation of *N*-(*tert*-Butyloxycarbonyl)-3-methyldehydroalanine Isopropyl Ester (BOC-MDHA-IPE). Three steps are involved in the preparation:

1. Preparation of Threonine Isopropyl Ester (Brenner et al., 1951). A 12-g portion of L-Thr was suspended in 250 mL of absolute isopropyl alcohol, dry HCl gas was passed into the suspension, and it was refluxed for 30 min. Isopropyl alcohol was removed under vacuum. The procedure was repeated by suspending the residue again in another 100 mL of isopropyl alcohol. HCl was removed by evaporating several times with 50 mL of isopropyl alcohol. The synthesized ester was liberated by dissolving the ester hydrochloride in 100 mL of chloroform and

0021-8561/89/1437-0304$01.50/0

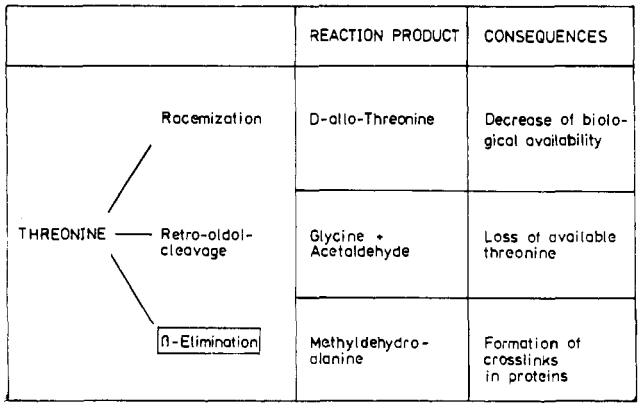

Figure 1. Reactions of Thr in alkaline medium.

adding a 2% ammoniacal chloroform solution. Ammonium chloride was filtered off, and the solvent was removed under vacuum.

2. Preparation of BOC-Threonine Isopropyl Ester. (Moroder et al., 1976). Threonine isopropyl ester (10 mmol) and triethylamine (10 mmol) were dissolved in 25 mL of dimethylformamide. Di-*tert*-butyl dicarbonate (11 mmol) was added, and the mixture was stirred for 10 min at room temperature. Solvent and triethylamine were evaporated to dryness on a rotary evaporator. A 20-mL portion of water was added, and the mixture was covered with a layer of 25 mL of ethyl acetate and adjusted to pH 2–3 with 5% $KHSO_4$. The organic phase was separated and the aqueous solution extracted twice with 25 mL of ethyl acetate. The organic phases were washed with distilled water and dried over Na_2SO_4, and the solvent was distilled off: yield 90.8%; purity 92%.

3. Dehydration of BOC-Threonine Isopropyl Ester with N,N'-Carbonyldiimidazole (Andruskiewicz and Czerwinski, 1982). A solution of the BOC-threonine isopropyl ester (5 mmol, 1.305 g), *N,N'*-carbonyldiimidazole (5 mmol, 0.81 g), and triethylamine (5 mmol, 0.7 mL) in dry tetrahydrofuran (20 mL) was stirred at room temperature for 6 h. The solvent was evaporated under vacuum. The substrate was purified by column chromatography on silica gel (70–230 mesh) and eluted with a toluene/dioxane mixture (9/1, v/v): ^{1}H NMR, δ 1.30 (d, 3 H, $OCHCH_3$), 1.57 (s, 9 H, $C(CH_3)_3$), 1.60 (d, 3 H, $C{=}CHCH_3$), 4.30 (d, 1 H, N*H*), 4.45 (dq, 1 H, $OCH(CH_3)_2$), 5.12 (mc, 1 H, $C{=}CHCH_3$); IR, 2970 (m), 1835 (s), 1740 (s), 1470 (m), 1365 (s), 1330 (s), 1215 (s), 1160 (s), 1100 (s), 1065 (s), 980 (m), 930 (m), 875 (w), 835 (w), 790 (w), 760 (w) cm^{-1}; MS, *m/z* 243 (M^+, 0.5), 187 (8), 145 (11), 128 (13), 127 (10), 101 (14), 100 (11), 83 (12), 57 (100), 43 (21). Abbreviations: s = singlet, d = doublet, t = triplet, m = multiplet, mc = center of a multiplet, dd = doublet of doublets, qui = quintet.

Preparation of LMeAL and LAL. LMeAL was synthesized by modifications of the methods of Okuda and Zahn (1965) and Fujimoto et al. (1981) originally used for the preparation of LAL: 124.9 mg (0.4 mmol) of BOC-MDHA-IPE and 76.8 mg (0.4 mmol) of *N*-α-acetyl-L-lysine were dissolved in a 4 mL of 0.2 M NaOH (0.8 mmol) in Pyrex test tubes. Reaction conditions: 60 °C/48 h; control reaction, thin-layer chromatography (TLC) after 24 and 48 h. TLC conditions: stationary phase, KG 60 F_{254}; mobile phase, butanol/acetic acid/water (4/1/1, v/v/v); hydrolysis with 4 mL of concentrated HCl at 110 °C/16 h. The mixture was evaporated to dryness several times with doubly distilled water on a rotary evaporator. The residue was taken up with 0.05 M HCl and again controlled by TLC. The reaction mixture was purified by means of a phosphorus cellulose ion exchanger. Conditions: elution reagent, HCl solution; linear concentration gradient, 0.05 to 0.3 M HCl (475 mL) with attached fraction collector; LMeAL eluted between 120 and 150 mL. The fractions containing LMeAL were collected and evaporated to dryness several times with doubly distilled water on a rotary evaporator: yield 5%; purity 83%. LMeAL, ^{1}H NMR: δ 1.37 (d, 3 H, H-4'), 1.42–159 (m, 2 H, H-4), 1.78 (qui, 2 H, H-5), 1.94 (mc, 2 H, H-3), 3.22 (dt, 2 H, H-6), 3.72 (mc, 1 H, H-3'), 3.89 (t, 1 H, H-2), 4.20 (d, 1 H, H-2'). LMeAL derivative, MS: *m/z* 532 (10), 490 (15), 418 (10), 407 (35), 376 (10), 365 (100), 180 (61).

The preparation of LAL resulted from 2-acetamidoacrylic acid with *N*-α-acetyl-L-lysine, according to the method of Fujimoto et al. (1981); 26 mg (0.2×10^{-3} mmol) of 2-acetamidoacrylic acid and 38 mg (0.2×10^{-3} mmol) of *N*-α-acetyl-L-lysine were dissolved in 2 mL of 0.2 M NaOH in Pyrex test tubes. Further reaction conditions are the same as those described for LMeAL. Hydrolysis was done with 2 mL of concentrated HCl.

Derivatization. Derivatization of LMeAL to its trifluoroacetic acid isopropyl ester (*N*-TFA-LMeAL-IPE) was necessary for the GC–MS analysis. It was conducted in 4-mL screw-cap glass flasks. The dry sample was derivatized with 1 mL of HCl–isopropyl alcohol (1.5 M HCl) for 1 h at 100 °C. The mixture was dried under nitrogen; 100 μL of trifluoroacetic acid anhydride was added. After 30 min at room temperature, the solution was again dried under nitrogen and dissolved in dichloromethane. *N*-TFA-LMeAL-IPE was ready for GC–MS analysis.

Peptide Reactions. Portions of 5 mg of the respective di- or tripeptides and 10 mg of *N*-α-acetyl-L-lysine were dissolved in alkaline buffer solutions (pH 9, sodium tetrahydroborate/hydrochloric acid; pH 11, disodium hydrogen phosphate/sodium hydroxide; pH 13, sodium hydroxide/potassium chloride). The reaction was carried out in Pyrex, screw-cap test tubes for 24 h at 80 °C in a thermostated water bath and subsequent hydrolysis with 6 M HCl for 4 h at 145 °C was carried out according to Roach and Gehrke (1970). The mixture was evaporated to dryness several times with doubly distilled water on the rotary evaporator. The hydrolysates were dissolved in 1 mL of sodium citrate buffer (pH 2.2) and analyzed by amino acid analysis.

RESULTS AND DISCUSSION

1. Preparation of LMeAL. LMeAL was synthesized by modifications of the methods of Okuda and Zahn (1965) in the first part and Fujimoto et al. (1981) in the second part of the reaction. Both methods were originally used for the preparation of LAL. The ϵ-amino group of Lys reacts with the double bond of MDHA analogous to a base-catalyzed Michael addition.

The method of preparation of LMeAL, analogous to the synthesis of LAL, is based on a two-step procedure. In the first step, MDHA is formed intermolecularly through β-elimination of Thr. In the second step, in the particular case of LMeAL, the ϵ-amino group of Lys attaches to the double bond of MDHA (Figure 2).

MDHA has to be prepared in the form of a stable derivative. The BOC-MDHA-IPE was synthesized from threonine isopropyl ester after derivatization with a BOC protecting group and dehydration with *N,N'*-carbonyldiimidazole. The product was purified by means of adsorption chromatography. The ^{1}H NMR, IR, and MS spectra were consistent with the assigned structure.

The preparation of LMeAL resulted from BOC-MDHA-IPE after reaction with *N*-α-acetyl-L-lysine. The product was hydrolyzed and purified by a phosphorus cellulose ion exchanger. The structure of LMeAL was

Table I. Formation of LMeAL in Peptides (%)

pH	Thr-Phe	Lys-Thr-Tyr with Ac-L-lys	Lys-Thr-Tyr without Ac-L-lys	Glu-Thr-Tyr	Thr-Lys-Tyr	Thr-Val-Leu
9	n tr	n tr	n tr	n tr	n tr	n tr
11	n tr	<0.1	<0.1	<0.1	n tr	n tr
13	n tr	9.2	3.8	2.3	n tr	n tr

[a] n tr = not traceable.

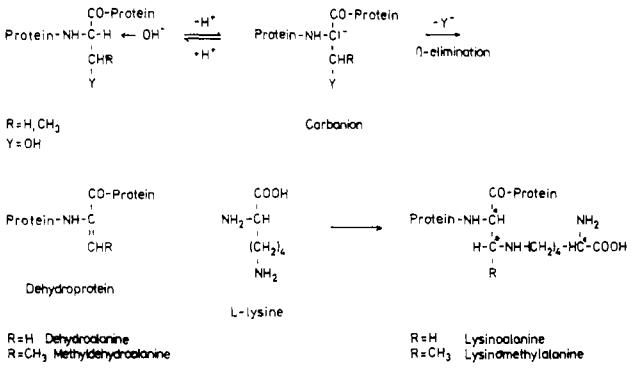

Figure 2. β-Elimination reaction.

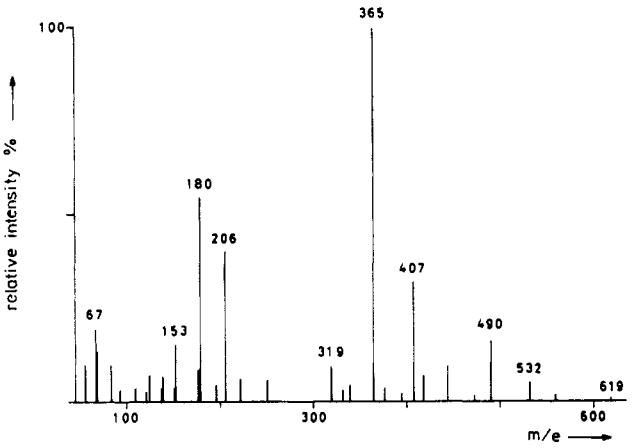

Figure 3. Mass spectrum of the *N*-TFA-IPE of LMeAL.

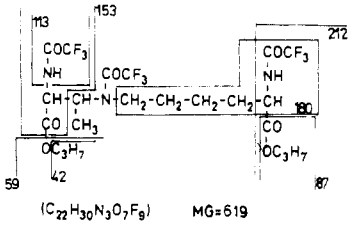

Figure 4. Fragmentation pattern of the *N*-TFA-IPE of LMeAL.

proved by ^{1}H NMR and GC–MS analysis.

The mass spectrum of the *N*-TFA-IPE of LMeAL is presented in Figure 3. The fragmentation pattern for the *N*-TFA-IPE of LMeAL is shown in Figure 4. The fragmentation characteristics are nearly identical with those of the corresponding LAL derivative (Hasegawa and Iwata, 1982). The base peak of LMeAL arises from the same decomposition reactions (M − 212 − 42) as reported for LAL and shows a difference of m/e 14 to the base peak of LAL. The NMR data correspond to the structure expected. The lysyl part of the molecule corresponds to the data given by Fujimoto et al. (1981).

Analysis of the TFA-LMeAL-IPE by capillary GC showed four closely consecutive peaks corresponding to four diastereomers of LMeAL (Figure 5). Since the molecule contains three chiral centers (Figure 2), eight

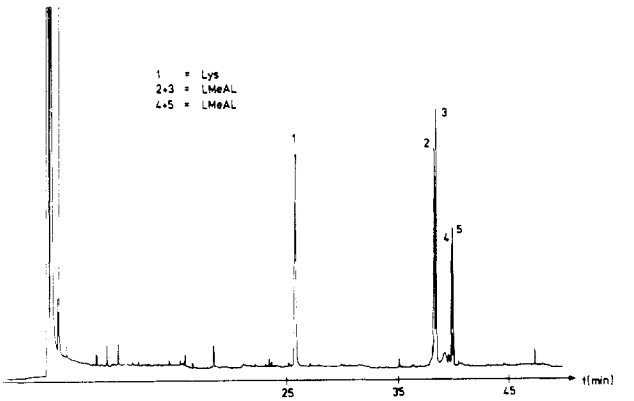

Figure 5. Gas chromatographic resolution of the *N*-TFA-IPE of LMeAL.

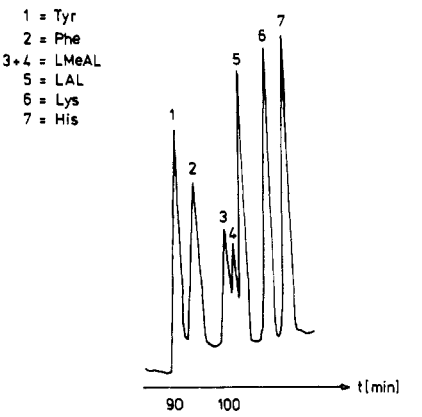

Figure 6. Elution position of LMeAL on an amino acid analyzer.

stereoisomers (four pairs of enantiomers) are possible. From the reaction of MDHA with L-Lys, four diastereomers are possible. Because of the alkaline reaction medium, partial racemization of Lys occurs (Provansal et al., 1975) and may lead to four pairs of enantiomers. LAL, which was also synthesized, showed two peaks by GC, corresponding to two diastereomers under the alkaline conditions used. Büser (1986) also found the two diastereomers of LAL by GC.

The LMeAL fraction showed two peaks by amino acid analysis eluting directly before LAL. Each peak presumably corresponds to two diastereomers of LMeAL shown in Figure 5. The cross-links LMeAL and LAL appeared in the amino acid chromatogram between Phe and Lys (Figure 6).

2. Formation of LMeAL in Peptides. Five peptides were examined in order to show the formation of LMeAL in the presence of L-Lys under alkaline conditions. Reactions were executed at pH 9, 11, and 13 for 24 h at 80 °C. The alkaline conditions were prepared with buffer solutions. The dipeptides Thr-Phe and Ser-Phe were investigated in order to determine differences between the formation of LMeAL and LAL. The tripeptides Lys-Thr-Tyr and Glu-Thr-Tyr with Thr in the central position and Thr-Lys-Tyr and Thr-Val-Leu with Thr at the N-

terminal position were used in order to show a potential influence of the primary structure on the amount of LMeAL. Reaction was carried out with N-α-acetyl-L-lysine added in each case. Reaction of the tripeptide Lys-Thr-Tyr was also executed without adding N-α-acetyl-L-lysine in order to show whether intramolecular formation of LMeAL is possible. The results of these experiments are shown in Table I.

At pH 9 no LMeAL appeared in the amino acid chromatogram for any of the peptides. Traces of LMeAL (<0.1%) could be detected at pH 11, but up to 10% of the theoretical possible amount appeared at pH 13.

Independent of pH conditions no LMeAL was formed in di- or tripeptides in which Thr is in the terminal position. In contrast with the dipeptide Ser-Phe as substrate, however, small quantities of LAL were formed at pH 11 and 13. This result reveals a different reactivity of Ser and Thr that may be due to the $+I$ effect of the methyl group in the MDHA. This $+I$ effect may somewhat stabilize the MDHA.

Detectable amounts of LMeAL could be demonstrated when Thr is in the middle position of the tripeptide (Lys-Thr-Tyr, Glu-Thr-Tyr), especially at pH 13. There are differences of amounts of LMeAL at pH 13, which indicate that the position of Thr and possibly the neighboring amino acids may influence the formation of LMeAL. Further studies are necessary in order to show the influence of the peptide sequence on the formation of LMeAL also in peptides with more than three amino acids.

Another question was whether an intramolecular formation of LMeAL or the formation of cross-links between peptides is possible. To prove this hypothesis, the peptide Lys-Thr-Tyr was treated without adding N-α-acetyl-L-lysine to the reaction mixture. The results of these experiments show clearly that intramolecular formation of LMeAL or the formation of cross-links is possible at both pH 11 and pH 13. Further experiments have to be conducted in order to show how many and what amino acids in peptides between Lys and Thr influence the amount of LMeAL formation. The results of the reported experiments do not reveal, however, whether the reactions of Lys and Thr took place intra- or/and intermolecularly. [^{15}N]Lys and [^{15}N]Thr in peptides may help in future experiments to distinguish between the two reactions.

The formation of LMeAL in proteins has been supposed by Lee et al. (1977). These authors, however, could not clearly prove the existence of LMeAL because a standard of LMeAL was not available.

Registry No. LMeAL, 69677-81-2; BOC-MDHA-IPE, 118869-99-1; L-Thr, 72-19-5; Thr-Phe, 16875-27-7; Ser-Phe, 16875-28-8; Lys-Thr-Tyr, 108191-44-2; Glu-Thr-Tyr, 110642-78-9; Thr-Lys-Tyr, 41961-62-0; Thr-Val-Leu, 66317-22-4; BOC-threonine isopropyl ester, 118869-98-0.

LITERATURE CITED

Andruszkiewicz, R.; Czerwinski, A. Dehydration of β-Hydroxyamino Acids with N,N'-Carbonyldiimidazole. *Synthesis* **1982**, 968–969.

Asquith, R. S.; Otterburn, M. S. Cysteine-Alkali Reactions in relation to Protein crosslinking. *Adv. Exp. Med. Biol.* **1977**, *86B*, 93–122.

Bohak, Z. N$^{\epsilon}$-(DL-2-amino-2-carboxyethyl)-L-lysine, a new amino acid formed on alkaline treatment of proteins. *J. Biol. Chem.* **1964**, *239*, 2878–2887.

Brenner, M.; Sailer, E.; Rüfenacht, K. Synthese des Threonin-isopropylesters. *Helv. Chim. Acta* **1951**, *34*, 2100.

Büser, W. Entwicklung eines gaschromatographischen Bestimmungsverfahrens für Aminosäuren und Hitzeschädigungsprodukte des Lysins. Dissertation, The University of Kiel, FRG, 1986.

Carroll, K. K. Review of clinical studies on cholesterol lowering response to soy protein. *Abstracts of Papers*; 70th Annual Meeting of the American Dietetic Association Atlanta, GA; The American Dietetic Association: Atlanta, GA, 1987.

de Groot, A. P.; Slump, P. Effects of severe alkali treatment of proteins on amino acid composition and nutritive value. *J. Nutr.* **1969**, *98*, 45–56.

Finot, P. A. Lysinoalanine in Food Proteins. *Nutr. Abstr. Rev.* **1983**, *53*, 67–80.

Friedman, M. Reactions of Proteins with Dehydroalanines. *Adv. Exp. Med. Biol.* **1977**, *86B*, 213–224.

Fujimoto, D.; Hirama, M.; Iwashita, T. Occurence of Lysinoalanine in calcified tissue collagen. *Biochem. Biophys. Res. Commun.* **1981**, *103*(4), 1378–1383.

Gould, D. H.; MacGregor, J. T. Biological effects of alkali-treated protein and lysinoalanine: an overview. *Adv. Exp. Med. Biol.* **1977**, *86A*, 29.

Hasegawa, K.; Iwata, S. Gas Chromatography-Mass Spectrometry of Lysinoalanine and the Related Crosslinked Amino Acids in Alkali-treated Food Proteins. *Agric. Biol. Chem.* **1982**, *46*, 2513–2520.

Lee, H. S.; Osuga, D. T.; Nashef, A. S.; Ahmed, A. I.; Whitaker, J. R.; Feeney, R. E. Effects of Alkali on Glykoproteins. β-Elimination and Nucleophilic Addition Reactions of Substituted Threonyl Residues of Antifreeze Glycoprotein. *J. Agric. Food. Chem.* **1977**, *25*, 1153–1158.

Liardon, R.; Hurrell, R. F. Amino Acid Racemization in Heated and Alkali-Treated Proteins. *J. Agric. Food Chem.* **1983**, *31*, 432–437.

Maga, J. A. LAL in Foods. *J. Agric. Food. Chem.* **1984**, *32*, 955–964.

Moroder, L.; Hallett, A.; Wünsch, E.; Keller, O.; Wersin, G. Di-tert.-butyldicarbonat - ein vorteilhaftes Reagenz zur Einführung der tert.-Butyloxycarbonyl-Schutzgruppe. *Hoppe-Seyler's Z. Physiol. Chem.* **1976**, *357*, 1651–1653.

Okuda, T.; Zahn, H. Synthese von N$^{\epsilon}$-[2-Amino-2-carboxy-äthyl]-L-lysin, einer neuen Aminosäure aus alkalibehandelter Wolle. *Chem. Ber.* **1965**, *98*, 1164–1167.

Provansal, M. M. P.; Cuq, J.-L. A.; Cheftel, J.-C. Chemical and Nutritional Modifications of Sunflower Proteins due to Alkaline Processing. Formation of Amino Acid Cross-Links and Isomerization of Lysine Residues. *J. Agric. Food Chem.* **1975**, *23*, 938–943.

Roach, D.; Gehrke, W. The Hydrolysis of Proteins. *J. Chromatogr.* **1970**, *52*, 393–404.

Woodard, J. C.; Alvarez, M. R. Renal lesions in rats fed diets containing α-protein. *Arch. Pathol.* **1967**, *84*, 153–162.

Received for review January 27, 1988. Revised manuscript received July 28, 1988. Accepted August 3, 1988.