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QUANTITATION OF THE ε-(γ-GLUTAMYL)LYSINE CROSS-LINK USING A HIGH-SPEED AMINO ACID ANALYZER WITHOUT PURIFICATION OF THE DIPEPTIDE

APPLICATION TO ENZYMATIC DIGESTED MIXTURES OF KERATIN AND THE MEMBRANOUS FRACTION OF HUMAN STRATUM CORNEUM

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SUMMARY

ε-(γ-Glutamyl)lysine in an enzymically digested mixture of keratin and the membranous fraction of human stratum corneum was directly quantitated using a high-speed amino acid analyzer without purification of the dipeptide. The analytical conditions were improved so that ε-(γ-glutamyl)lysine clearly separated from other amino acids and eluted directly after tyrosine. The enzymatically digested mixtures were filtered through an Ultra Free membrane and deammoniated before analysis. By our present method, keratin and the membranous fraction of human stratum corneum were analyzed and 5.8 and 43.5 nmol/mg of ε-(γ-glutamyl)lysine, respectively, were detected.

INTRODUCTION

ε-(γ-Glutamyl)lysine is known to be one of the cross-links within and between molecules [1]. It is formed by the catalytic action of transglutaminase, which is a Ca^{2+}-dependent acyl transfer reaction between peptide-bound glutamine residues and peptide-bound lysine residues. The role of this

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covalent cross-link is to maintain gross forms of structure and limits of degrees of extensiveness. Pisano et al. [2] detected the ϵ-(γ-glutamyl)lysine cross-link in Factor XIII (plasma transglutaminase) polymerized fibrin after chemical and enzymatic treatments. Mosher [3] reported that fibronectin is a substrate for Factor XIII and can be cross-linked to collagen and the α-chain of fibrin. Birckbicher et al. [4] reported that there was less transglutaminase activity, fibronectin and ϵ-(γ-glutamyl)lysine in malignant hepatoma, virus-transformed human and hamster cells than in normal counterparts. Abernethy et al. [5] found ϵ-(γ-glutamyl)lysine cross-links in human stratum corneum and determined the dipeptide.

Following these analytical studies, many authors attempted to detect the cross-link in numerous tissues, organelles and proteins. Conventional methods are as follows. High molecular weight proteins or cell membranes which might contain ϵ-(γ-glutamyl)lysine cross-links were digested by many kinds of exo- and endopeptidases to determine the dipeptide. After digestion, the cross-linked dipeptide was purified using column liquid chromatography or electrophoresis. Finally, the dipeptide that consisted of glutamyl and lysyl residues was identified using amino acid analysis after acid hydrolysis. These purification and identification processes were complicated and time-consuming.

In this paper, we demonstrate ϵ-(γ-glutamyl)lysine cross-links in enzymically digested mixtures of keratin and the membranous fraction of human stratum corneum using a high-speed amino acid analyzer without purification of the dipeptide.

EXPERIMENTAL

Reagents

ϵ-(γ-Glutamyl)lysine was purchased from Vega-Fox Biochemicals (lot No. 10883) and α-aminobutyric acid from Sigma. A standard solution of ninhydrin-positive compounds was prepared as described in a previous paper [6]. Subtilisin, pronase, carboxypeptidase A-diisopropylfluorophosphate (DFP), leucine aminopeptidase and prolidase were the products of Sigma. Carboxypeptidase B was purchased from Boehringer Mannheim.

TABLE I

EXPERIMENTAL CONDITIONS FOR ϵ-(γ-GLUTAMYL)LYSINE

Column: 2.6 × 250 mm stainless-steel column packed with Hitachi ion-exchange resin No. 2619.

Buffers: (1) 0.155 *N* lithium citrate pH 3.0 (lithium citrate 9.80 g/l, LiCl 2.12 g/l, citric acid 35 g/l, ethanol 40 ml/l, thiodiglycol 5 ml/l, and 25% Brij-35 4 ml/l).
(2) 0.255 *N* lithium citrate pH 3.7 (lithium citrate 9.80 g/l, LiCl 6.36 g/l, citric acid 13 g/l, ethanol 30 ml/l, thiodiglycol 5 ml/l, and 25% Brij-35 4 ml/l).
(3) 0.200 *N* LiOH (LiOH 8.40 g/l, and 25% Brij-35, 4 ml/l).

Flow-rates: Buffer pump 0.275 ml/min, 190 kg/cm^2.
Ninhydrin pump 0.300 ml/min, 30 kg/cm^2.

Programs:									
Buffer change times	0		70		140		148		180 min
		Buffer 1		Buffer 2		Buffer 3		Buffer 1	
Column temperature	0		32		81				180 min
		34°C		43°C		34°C			

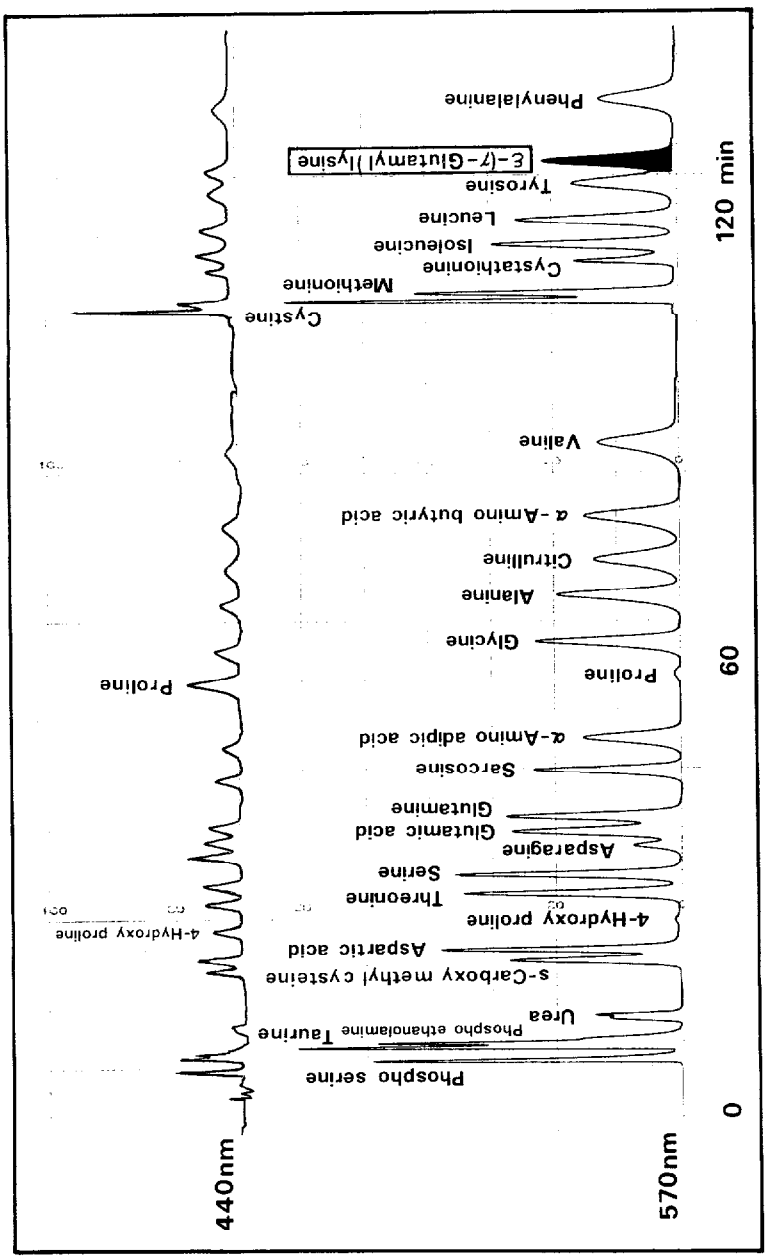


Fig. 1. Chromatogram of ε-(γ-glutamyl)lysine and amino acids using a high-speed amino acid analyzer. The concentration of ε-(γ-glutamyl)lysine was 1.463 nmol and that of other amino acids was 2.5 nmol except for cystathionine (1.0 nmol), S-carboxymethylcysteine (1.25 nmol), proline (5.0 nmol), sarcosine (5.0 nmol) and urea (250 nmol).

Analysis of amino acids and ε-(γ-glutamyl)lysine

Previously we reported an improved method for analyzing physiological amino acids using an Hitachi 835 high-speed amino acid analyzer [7]. Based on this analytical method, further modifications were made in order to identify ε-(γ-glutamyl)lysine. The detailed conditions are given in Table I.

Purification of ε-(γ-glutamyl)lysine as a standard

We had purchased ε-(γ-glutamyl)lysine from Vega-Fox Biochemicals; however, many peaks besides the main peak emerged on the amino acid chromatogram. Therefore, the ε-(γ-glutamyl)lysine was purified from this commercial source by thin-layer chromatography (TLC). The TLC plate pre-coated with silica gel was purchased from Merck. *n*-Butanol—acetic acid—water—ethyl acetate (1:1:1:1) was the solvent. The amino acids and ε-(γ-glutamyl)lysine were detected by spraying with a ninhydrin reagent. There were two spots, with R_F values of 0.25 and 0.20. The upper spot (R_F 0.25) was collected and extracted with 0.01 *N* hydrochloric acid. This spot was a single peak on the amino acid chromatogram and was found to contain equimolar amounts of glutamic acid and lysine after hydrolysis with 6 *N* hydrochloric acid at 110°C for 24 h.

Enzymic digestion of keratin and the membranous fraction of human stratum corneum

Keratin was prepared from human stratum corneum according to the method of Ogawa and Hattori [8]. The membranous fraction was isolated from the same materials, as described in our previous paper [9]. Sulfide bonds were modified by carboxymethylation as described by Abernethy et al. [5]. Each 25 mg of the carboxymethylated keratin and membranous fraction was digested with, sequentially, subtilisin (2%), pronase (2%), carboxypeptidase A (2%) and B (0.5%), leucine aminopeptidase (2%), prolidase (2%) and leucine

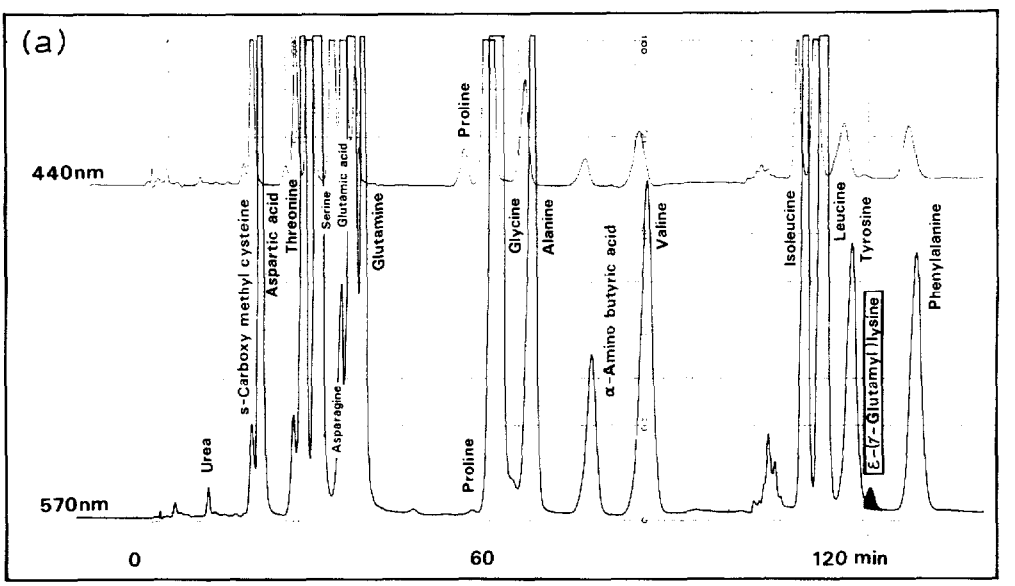


Fig. 2.

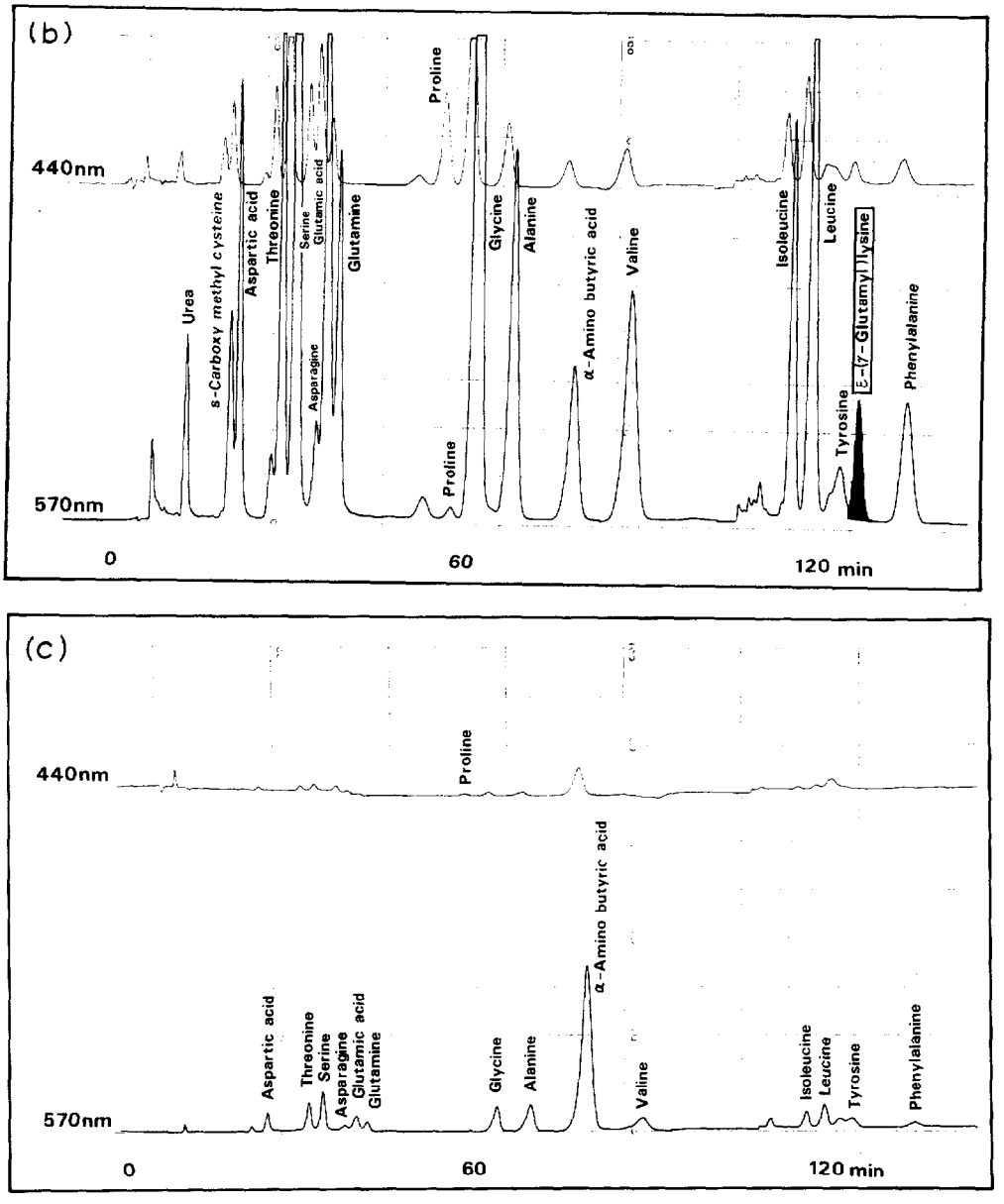


Fig. 2. Chromatograms of keratin (a) and the membranous fraction (b) of human stratum corneum for ϵ-(γ-glutamyl)lysine. Chromatogram (c) was obtained from the enzyme mixture as a control (10.8 μl). The amounts of keratin and the membranous fraction were 39.9 and 39.5 μg, respectively.

aminopeptidase (2%) using a modification of the method of Abernethy et al. [5]. We performed each enzymic digestion for 24 h at 37°C. Finally, digested mixtures were filtered through an Ultra Free membrane (Millipore), adjusted to pH 12 with 1 *N* sodium hydroxide solution and deammoniated under vacuum for about 12 h.

RESULTS

Separation of ε-(γ-glutamyl)lysine on the amino acid chromatogram

A sample was made by mixing purified ε-(γ-glutamyl)lysine (as described under Experimental) with the amino acid solution. Then, we improved the experimental conditions of amino acid analysis for clear detection of ε-(γ-glutamyl)lysine using a high-speed amino acid analyzer. Fig. 1 shows the chromatogram of ε-(γ-glutamyl)lysine and the other amino acids. ε-(γ-Glutamyl)-lysine clearly separated from the other amino acids and appeared just behind the tyrosine peak. It took 126.22 min. The resolution between tyrosine and the dipeptide was more than 90%. Following this result, ε-(γ-glutamyl)lysine in keratin and the membranous fraction was analyzed using the high-speed amino acid analyzer. Fig. 2 shows the chromatograms obtained with the enzymically digested mixtures of these fraction.

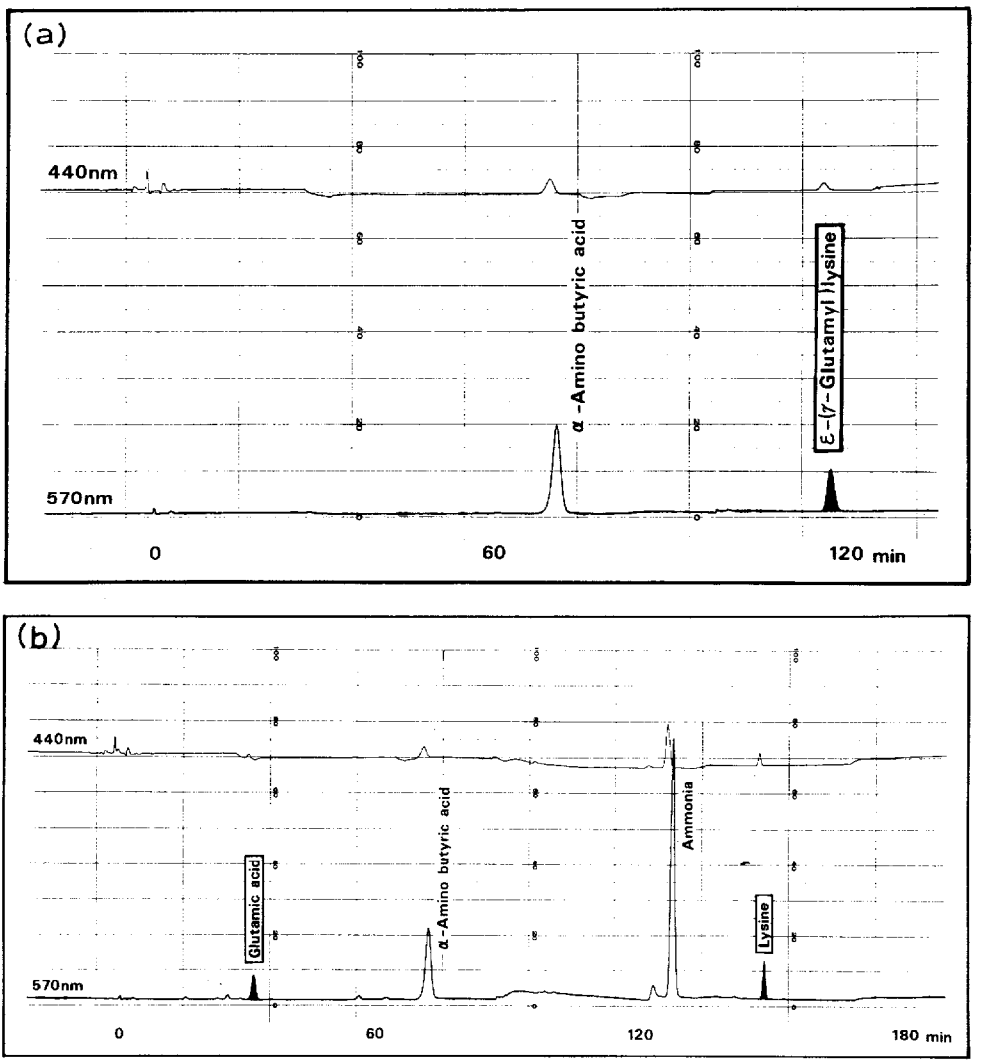
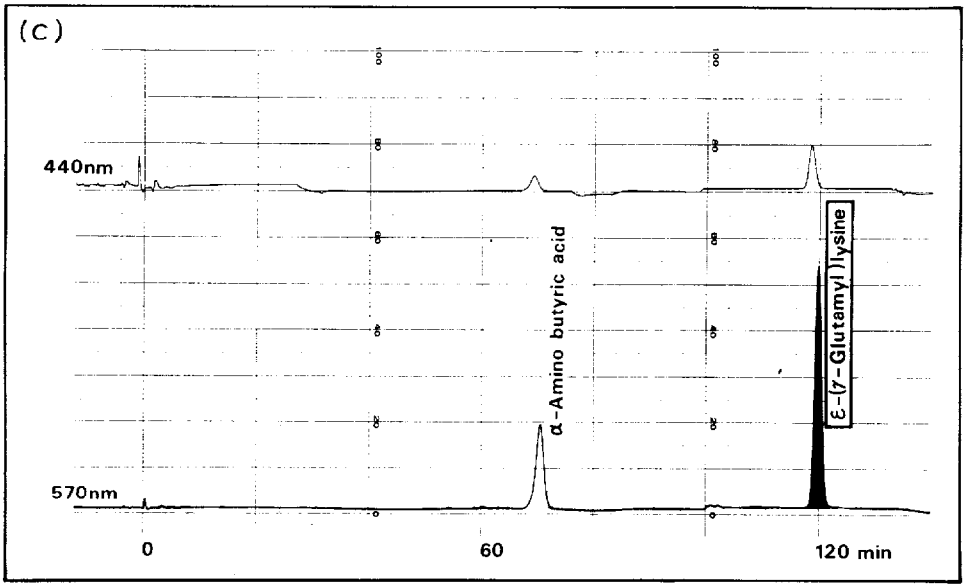


Fig. 3.



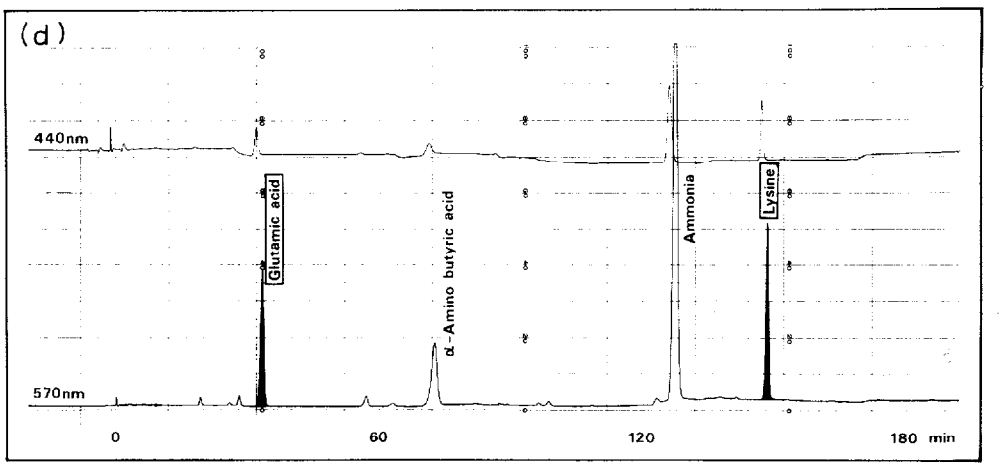


Fig. 3. (a) Chromatograms of ε-(γ-glutamyl)lysine obtained from the membranous fraction of human stratum corneum; and (b) after hydrolysis. (c) Chromatogram of ε-(γ-glutamyl)-lysine obtained from the standard solution (2.5 nmol); and (d) after hydrolysis.

Keratin contained a small amount of the dipeptide and its dipeptide peak was resolved about 50% from the tyrosine peak. The membranous fraction contained much more dipeptide and its dipeptide peak was completely resolved from the tyrosine peak.

Further identification of ε-(γ-glutamyl)lysine in the membranous fraction

We ascertained whether or not the peak behind tyrosine on the chromatogram of the membranous fraction of human stratum corneum was only ε-(γ-glutamyl)lysine. We used a manual sampler attached to a Hitachi 835 high-

speed amino acid analyzer. A 100-μl volume of the enzymatic digested mixture (362 μg of membranous fraction) was charged on the column. The effluent corresponding to the retention time of ϵ-(γ-glutamyl)lysine was collected before addition of ninhydrin reagent. α-Aminobutyric acid (10 nmol per 100 μl) as an internal standard was added to part (100 μl) of that effluent. Seventy microliters of the mixture were directly charged on the column again. The other 100 μl were hydrolyzed with 6 *N* hydrochloric acid at 110°C for 24 h and the amino acid composition determined. Fig. 3 shows the chromatograms of ϵ-(γ-glutamyl)lysine eluted from the membranous fraction with (Fig. 3a) and without (Fig. 3b) hydrolysis. There are two major peaks on the chromatogram before hydrolysis. The faster peak is the internal standard and the later one is ϵ-(γ-glutamyl)lysine. After hydrolysis, glutamic acid and lysine emerged in almost equimolar amounts and ϵ-(γ-glutamyl)lysine disappeared (Fig. 3b). We calculated the molar ratios of ϵ-(γ-glutamyl)lysine/glutamic acid/lysine as 1:0.95:0.91. Large amounts of ammonia and another small peak appeared on the chromatogram after hydrolysis. They were also found on the chromatogram where ϵ-(γ-glutamyl)lysine as the standard substance was eluted from the column and hydrolyzed under the same conditions (Fig. 3d).

Pre-treatment of the enzymically digested mixture before amino acid analysis

A large amount of ammonia was produced during the enzymic digestions. The following pre-treatments were carried out before amino acid analysis: the digested mixture was (A) centrifuged at 1050 *g* for 5 min, (B) centrifuged under the same conditions and deammoniated at pH 12 under vacuum, (C) filtered through an Ultra Free membrane, and (D) filtered through the membrane and deammoniated by the same method. Fig. 4 shows the chromatograms of the samples treated by procedures A, B, C and D. The peak areas of methionine, tyrosine, phenylalanine and ϵ-(γ-glutamyl)lysine from each chromatogram are shown in Table II.

During deammoniation, the peak areas of tyrosine and methionine decreased markedly, whereas those of phenylalanine and ϵ-(γ-glutamyl)lysine were not so

TABLE II

COMPARISON OF PRE-TREATMENTS ON THE ENZYMICALLY DIGESTED MIXTURE OF THE MEMBRANOUS FRACTION OF HUMAN STRATUM CORNEUM

Pre-treatment	nmol/mg membranous fraction			
	(A) Centrifugation	(B) Centrifugation + deammoniation	(C) Filtration	(D) Filtration + deammoniation
Methionine	23.0	2.1	24.4	4.9
Tyrosine	146.0	92.7	140.9	37.1
Phenylalanine	114.1	118.8	118.3	121.8
ϵ-(γ-Glutamyl)lysine	40.1	39.1	40.4	38.7
Total amino acids after enzymic digestion	4269 (87%)	4195 (86%)	4306 (88%)	4191 (87%)
Total amino acids after hydrolysis*	4906 (100%)	4898 (100%)	4900 (100%)	4838 (100%)

*6 *N* Hydrochloric acid at 110°C for 24 h.

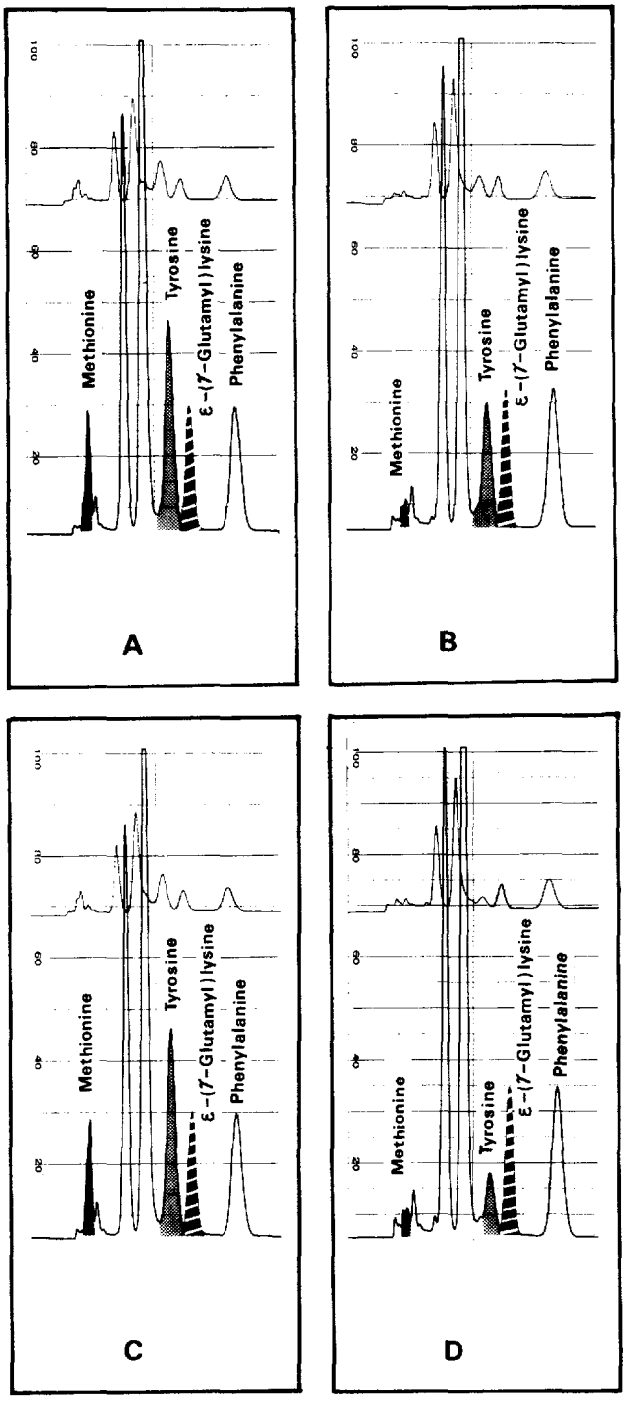


Fig. 4. Comparison of pre-treatments for ε-(γ-glutamyl)lysine in the membranous fraction. (A) Centrifugation, (B) centrifugation + deammoniation, (C) filtration, and (D) filtration + deammoniation.

variable. The former two peaks overlapped the ammonia peak which derived from the sample during the enzymic digestion.

The quantitative analysis of ε-(γ-glutamyl)lysine was affected by tyrosine. It was necessary, therefore, for the enzymically digested mixture to be pre-treated by deammoniation. The mixture was also filtered through the Ultra Free membrane to reject a small number of large molecules.

The total amino acid values of each pre-treated sample with and without acid hydrolysis are compared in Table II. These values were not so variable in all cases. In the membranous fraction, the amounts of amino acids released by enzymic digestion were over 85% (86—88%) of the acid hydrolyzed one.

Quantitation of ε-(γ-glutamyl)lysine in keratin and the membranous fraction of human stratum corneum

We determined ε-(γ-glutamyl)lysine in keratin and the membranous fraction of human stratum corneum digested by various enzymes according to a modified method of Abernethy et al. The results are shown in Table III. There were small amounts of ε-(γ-glutamyl)lysine in keratin, whereas the membranous fraction contained about seven times as much as keratin did.

TABLE III

QUANTITATION OF ε-(γ-GLUTAMYL)LYSINE IN KERATIN AND THE MEMBRANOUS FRACTION OF HUMAN STRATUM CORNEUM

	nmol/mg material	
	Keratin	Membranous fraction
Experiment 1	4.9	43.3
Experiment 2	6.7	43.6
Average	5.8	43.5

DISCUSSION

We have modified the conditions of a high-speed amino acid analyzer to identify ε-(γ-glutamyl)lysine, which overlapped the leucine peak when we followed our previous method [7]. The procedure was improved (Table I) so that the first and second buffer change times were longer than in the original method [7] and the column temperature was altered from 34 to 43 to 34°C. Under these conditions, ε-(γ-glutamyl)lysine appeared between tyrosine and phenylalanine (Fig. 1).

In order ascertain whether that peak contained other peptides, the ε-(γ-glutamyl)lysine fraction eluted from the high-speed amino acid analyzer was hydrolyzed with 6 *N* hydrochloric acid and measured with the same analyzer. Small amounts of ninhydrin-positive compounds, apart from glutamic acid, lysine and the internal standard, appeared after hydrolysis. They were also detected only in the buffer solution eluted from the same instrument after acid hydrolysis. Thus it seems that these peaks were derived from the citrate buffers

or the column used with the high-speed amino acid analyzer. Even if polypeptides were included in that peak, they did not appear on the chromatogram, because the time taken for reaction with the ninhydrin reagent, 1.70 min, was too short for the polypeptides to react.

It was necessary for the enzymically digested mixture to be deammoniated. Ammonia was produced from glutamine and asparagine during enzymic digestion. Large amounts of ammonia overcharged the column and overlapped the tyrosine peak on the amino acid chromatograms.

The membranous fraction of human stratum corneum contained a large amount of ϵ-(γ-glutamyl)lysine in comparison with keratin. It also had a number of disulfide bonds [9]. It seems that the membranous fraction of the stratum corneum has the strict structure necessary for these cross-links.

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