Improved Method for Identification and Determination of ε-(γ-Glutamyl)lysine Cross-Link in Protein Using Proteolytic Digestion and Derivatization with Phenyl Isothiocyanate followed by High-Performance Liquid Chromatography Separation

Kenji Sato,*,† Yasuyuki Tsukamasa,‡ Chiharu Imai,‡ Kozo Ohtsuki,† Yutaka Shimizu,§ and Makoto Kawabata†

Department of Food Science and Nutrition, Kyoto Prefectural University, Shimogamo, Kyoto 606, Japan, Central Research Institute, Marudai Food Company, Ltd., Midori, Takatsuki, Osaka 569, Japan, and Kobe-gakuin Women's Junior College, Nagata, Kobe 653, Japan

An improved method for the identification and determination of ε-(γ-glutamyl)lysine cross-link in food protein is described. The cross-link was liberated by exhaustive proteolytic digestion and derivatized with PITC. The derivatives were resolved by HPLC. The present method enables one to determine simultaneously protein-constituting amino acids and ε-(γ-glutamyl)lysine and thereby facilitates the monitoring of progress of proteolytic digestion. The PTC-ε-(γ-glutamyl)lysine peak was collected and confirmed by conversion to a stable PTH form followed by HCl hydrolysis. The present method was successfully applied for the identification and determination of the cross-link in sardine meat gel.

INTRODUCTION

It has been demonstrated that transglutaminases (EC 2.3.2.13) form ε-(γ-glutamyl)lysine cross-links between proteins [see review by Folk and Chung (1973)]. To improve nutritional and textural properties of many food proteins, exogenous transglutaminases have been used to form ε-(γ-glutamyl)lysine cross-links between proteins (Ikura et al., 1980a,b, 1981; Motoki and Nio, 1983; Kurth and Rogers, 1984; Nonaka et al., 1989; Tanaka et al., 1990; Kato et al., 1991). On the other hand, it has been also suggested that endogenous transglutaminases in fish meat might form ε-(γ-glutamyl)lysine cross-links in fish proteins and change textural properties of fish meat sol during processing (Seki et al., 1990; Tsukamasa and Shimizu, 1990, 1991; Kimura et al., 1991).

Highly sensitive determination of ε-(γ-glutamyl)lysine cross-links has been performed by liberation of the cross-link by exhaustive proteolytic digestion and derivatization with *o*-phthalaldehyde (OPA) followed by high-performance liquid chromatography (HPLC) separation (Griffin et al., 1982; Beninati et al., 1988; Nonaka et al., 1989; Tanaka et al., 1990; Kimura et al., 1991). It has been, however, demonstrated that the fluorescence intensity of OPA-2-mercaptoethanol derivatives depends on temperature and reaction time (Brückner et al., 1987). Moreover, the relative fluorescence of OPA-ε-(γ-glutamyl)-lysine decreases to 83% of its initial value after only 45 min in storage (Griffin et al., 1982). Consequently, optimum derivatization temperature and time must be maintained under those systems to overcome the instability of the OPA-2-mercaptoethanol derivatives.

Recently, an alternative method for the determination of ε-(γ-glutamyl)lysine based on derivatization with phenyl isothiocyanate (PITC) has been reported (Fesus et al., 1989; Tarcsa and Fesus, 1990). According to that system, however, the ε-(γ-glutamyl)lysine peak was not completely separated from the other PTC amino acid peaks. In addition, a two-step preliminary chromatography purification was unavoidable before derivatization.

In this paper, we describe an improved method for identification and determination of the ε-(γ-glutamyl)-lysine cross-link based on derivatization with PITC and HPLC separation without complicated preliminary separation. This method was successfully applied for the identification and determination of ε-(γ-glutamyl)lysine cross-link in the $CaCl_2$-added sardine meat gel.

MATERIALS AND METHODS

Fish Sample. Sardine (*Sardinops melanosticta*) was purchased from a local fish supplier. Fish was used within a 12-h period from the expiry time.

Chemicals. ε-(γ-Glutamyl)lysine was purchased from Sigma (St. Louis, MO). Amino acid standard mixtures (type H, A/N and B), asparagine, glutamine, glutamic acid, lysine, and phenyl isothiocyanate (PITC) were purchased from Wako (Osaka, Japan). Triethylamine (TEA, sequence grade) was purchased from Pierce (Rockford, IL). Acetonitrile (HPLC grade) and trifluoroacetic acid (TFA) were purchased from nacalai tesque (Kyoto, Japan). All other reagents were of analytical grade.

Proteolytic Enzymes. *Streptomyces griseus* protease (Pronase E, type XXV), porcine kidney leucine aminopeptidase (type III), prolidase, and bovine pancreas carboxypeptidase A (type II) were purchased from Sigma. Wheat carboxypeptidase W was purchased from Seikagaku Kogyo (Tokyo, Japan).

High-Performance Liquid Chromatography (HPLC) Apparatus. The HPLC apparatus consisted of a Shimadzu LC-9A pump, an FLC-9A gradient valve unit, a GU-2 degasser unit, an SPD-6AV variable-wavelength detector, a CR-6A integrator, and a Rheodyne sample injector, Model 7125. All were obtained from Shimadzu (Kyoto, Japan).

Preparation of Sardine Meat Gel. White muscle was excised from the dorsal part of the sardine trunk and minced by passing it through a 10-mesh screen. The minced muscle (20 g) was homogenized with 100 mL of sodium phosphate buffer, pH 7.5, of which the ionic strength was adjusted to 0.05. The homogenate was centrifuged at 3000*g* for 10 min. These procedures were repeated twice. Before final centrifugation, the homogenate was passed once again through the 10-mesh screen. The final precipitate was suspended in 20 mM Tris-maleate buffer, pH 7.5, containing 0.5 M KCl to give a final protein concentration of 1% (w/v). Five milliliters of the meal sol obtained was pi-

* Author to whom correspondence should be addressed.
† Kyoto Prefectural University.
‡ Marudai Food Co.
§ Kobe-gakuin Women's Junior College.

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petted into a polypropylene tube. When the sol was added with 50 μL of 100 mM $CaCl_2$ and incubated at 25 °C for 24 h, gel formation occurred. The gel thus obtained was immediately heated in a boiling water bath for 3 min and is referred to as the sardine meat gel in the following discussion. As a control, some sol was heated without the incubation with $CaCl_2$. After heating, $CaCl_2$ was added. Protein concentration was estimated by a microbiuret method. Bovine serum albumin was used as the standard.

HCl Hydrolysis of Protein. A protein sample (5 mg/0.5 mL) was mixed with an equal volume of 12 M HCl and adjusted to 10 mL by adding 6 M HCl. The sample was hydrolyzed at 150 °C in vacuo for 1 h. The glassware used for hydrolysis was prewashed with 6 M HCl at 150 °C for 2 h. This pretreatment allowed for high recovery of aspartic acid and glutamic acid.

Exhaustive Proteolytic Digestion. Proteolytic digestion of a protein sample (5 mg/0.5 mL) was carried out by the sequential addition of proteolytic enzymes directly to the reaction mixture at 37 °C in the presence of a piece of zymole crystal. Proteolytic condition was as follows: Pronase E (4.0 units/5 g of protein) for 24 h; mixture of leucine aminopeptidase (4.9 units), prolidase (27.8 units), and carboxypeptidase A (15.4 units) for 24 h; and finally carboxypeptidase W (19.5 units) for 24 h. Before the addition of carboxypeptidase W, the pH value of the sample solution was adjusted to 4.0 by adding 1 M HCl. The proteolytic digests thus obtained were adjusted to 10 mL with distilled water and clarified by centrifugation at 10000*g* for 15 min. The proteolytic enzymes were removed from the digest by ultrafiltration using an Ultrafree C3-LGC (Millipore, Milford, MA).

Precolumn Derivatization with PITC. Derivatization of amino acid with PITC was performed according to the method of Bidlingmeyer et al. (1984) with a slight modification. Amino acid standard mixture, acid hydrolysate, and proteolytic digest of protein sample were pipetted into a glass tube (50 mm × 5 mm i.d.), respectively. The tubes were placed in a reaction vial with a resealable enclosure which was obtained from Waters (Milford, MA). The tube contents were dried by vacuum. Ten microliters of the redrying solution, which consisted of methanol, water, and TEA in the ratio 2:1:1, was added to the tubes. After the contents were redried, 20 μL of the derivatizing solution, which consisted of methanol, water, TEA, and PITC in the ratio 7:1:1:1, was added. Then the tubes were covered with Parafilm and allowed to stand for 20 min at 25 °C for completion of derivatization. The excess reagent was also removed by vacuum.

Conversion of Phenylthiocarbamyl (PTC) Amino Acid and Peptide to Phenylthiohydantoin (PTH) Form. PTC amino acid and peptide were completely dried in a glass tube (50 mm × 5 mm i.d.) and converted to PTH form according to the method of Tarr (1986). Briefly, 10 μL of TFA was added to the tube and the tube flushed with nitrogen gas. After 5 min of incubation at 50 °C, TFA was removed by vacuum. Ten microliters of a mixture of 2 M HCl and methanol (1:1) was added and flushed with nitrogen gas. After 10 min of incubation at 50 °C, the reagent was removed by vacuum. PTH amino acid and ε-(γ-glutamyl)lysine thus obtained were hydrolyzed by vapor HCl at 150 °C in vacuo according to the method of Bidlingmeyer et al. (1984).

HPLC Separation of ε-(γ-Glutamyl)lysine and Amino Acid Derivatives. PTC and PTH amino acids and peptides were dissolved in 5 mM sodium phosphate buffer, pH 7.4, containing 10% (v/v) acetonitrile (Pico-Tag sample diluent), and the solution was filtered through a 0.45-μm filter (Columngard, Millipore, Bedford, MA). The filtrate was applied on a Cosmosil AR C_{18} column (250 mm × 4.6 mm i.d., particle size 5 μm, nacalai tesque, Kyoto, Japan), which can be obtained from JM Science (Buffalo, NY), using binary linear multistep solvent gradients. Solution A consisted of 150 mM ammonium acetate buffer, pH 6.0, containing 5% acetonitrile (for PTC amino acid) or 80 mM ammonium acetate buffer, pH 5.0, containing 5% acetonitrile (for PTH amino acid). Solution B consisted of acetonitrile and water (6:4). The gradient profiles were as follows: 0–2 min, 0% B; 2–20 min, 5–47.5% B; 20–25 min, 47.5–90% B; 25–35 min, 100% B; 35–45 min, 0% B for PTC amino acid; and 0–30 min, 10–70% B; 30–40 min, 100% B; 40–50 min, 0% B for PTH amino acid. Flow rate was 1 mL/min, and column temperature was maintained at 43 °C.

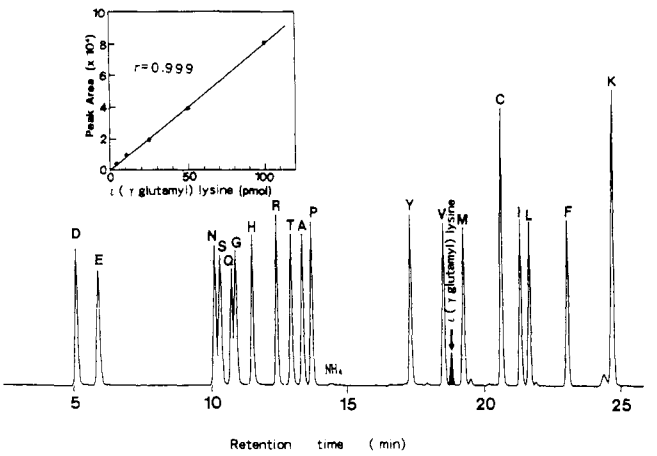

Figure 1. Chromatogram of PTC ε-(γ-glutamyl)lysine and protein-constituting amino acids. Peaks are labeled with one-letter abbreviations for protein-constituting amino acids: D, aspartic acid; E, glutamic acid; N, asparagine; S, serine; Q, glutamine; G, glycine; H, histidine; R, arginine; T, threonine; A, alanine; P, proline; Y, tyrosine; V, valine; M, methionine; C, cystine; I, isoleucine; L, leucine; F, phenylalanine; K, lysine. Detection was at 254 nm (0.160 AUFS). ε-(γ-Glutamyl)lysine and protein-constituting amino acids were present at 50 and 500 pmol, respectively. (Inset) Plot of peak area vs quantity of PTC ε-(γ-glutamyl)lysine.

RESULTS

Figure 1 shows a representative elution pattern of authentic ε-(γ-glutamyl)lysine and protein-constituting amino acids. Excellent resolution was achieved for all derivatives. Any amino acids present in amino acid standard physiological A/N and B (Wako) did not coelute with ε-(γ-glutamyl)lysine (data not shown). The relationship between peak area and quantity of ε-(γ-glutamyl)-lysine was found to be linear ($r = 0.999$) at the low picomole level (Figure 1, inset). Eight hundred femtomoles of ε-(γ-glutamyl)lysine peak could be detected at 0.04 AUFS without any interferences (data not shown). Then ε-(γ-glutamyl)lysine can be, at least, determined accurately at the picomole level. The elution patterns of the proteolytic digest and HCl hydrolysate samples from the sardine meat sol, which was not incubated with $CaCl_2$, are shown in Figure 2. In both cases, good resolutions were also achieved. The chromatogram of the proteolytic digest was comparable with that of the acid hydrolysate except for the presence of asparagine and glutamine. As shown in Table I, the amounts of lysine and the sum of glutamine and glutamic acid in the proteolytic digest were in good agreement with those of lysine and glutamic acid in the HCl hydrolysate, respectively. Then glutamyl and lysyl residues in sardine meat sol proteins were able to be liberated by the proteolytic digestion. Sufficient liberation of other amino acids except for glycine was also achieved by the proteolytic digestion. The incomplete liberation of glycine by the proteolytic digestion remained to be solved. However, it is not essential to the present study. Authentic ε-(γ-glutamyl)lysine (10 μmol/10 mL) was also treated with the proteolytic enzymes under the same conditions. As shown in Figure 3, ε-(γ-glutamyl)lysine was not destroyed by the proteolytic digestion. Only negligible amounts of protein-constituting amino acids were detected in the chromatogram. Then the higher recovery of some amino acids such as serine and threonine by the proteolytic digestion as compared to that by HCl hydrolysis might be due to the destruction of these amino acids by acid hydrolysis rather than the autolysis of proteolytic enzymes. ε-(γ-Glutamyl)lysine cross-links in

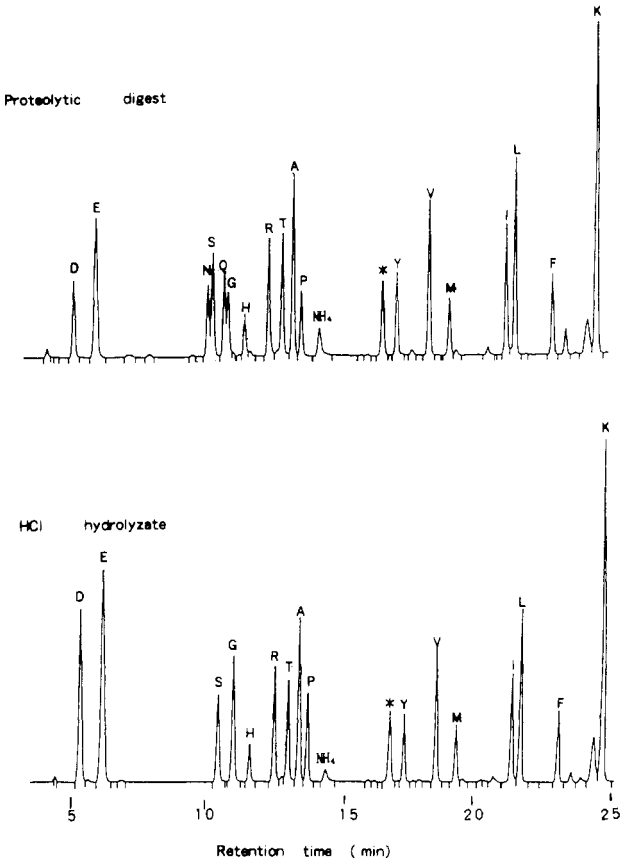

Figure 2. Chromatograms of proteolytic digest and HCl hydrolysate of sardine meat sol. Peak marked with the asterisk was derived from Tris buffer (0.160 AUFS). Refer to the legend of Figure 1 for abbreviations.

Table I. Amino Acid Composition of Proteolytic Digest and HCl Hydrolysate of Sardine Meat Sol

	nmol/μg of protein	
	proteolytic digest[a]	HCl hydrolysate[b]
aspartic acid	387 ± 62	881 ± 3
glutamic acid	856 ± 49	1343 ± 21
asparagine	337 ± 39	
serine	547 ± 50	426 ± 3
glutamine	489 ± 28	
glycine	345 ± 33	669 ± 9
histidine	202 ± 3	162 ± 1
arginine	505 ± 19	462 ± 10
threonine	616 ± 28	452 ± 8
alanine	811 ± 26	707 ± 16
proline	290 ± 7	350 ± 6
tyrosine	344 ± 2	251 ± 1
valine	642 ± 11	515 ± 13
methionine	266 ± 9	235 ± 5
isoleucine	540 ± 9	431 ± 11
leucine	808 ± 16	677 ± 21
phenylalanine	337 ± 6	283 ± 1
lysine	813 ± 25	768 ± 29

[a] Means ± SD (n = 4). [b] Means ± SD (n = 2).

sardine meat protein can be liberated by the proteolytic digestion and the liberated ε-(γ-glutamyl)lysine can be determined by derivatization with PITC and reversed-phase HPLC separation.

PTC ε-(γ-glutamyl)lysine dissolved in the Pico-Tag diluent solution was kept at 4 °C. After 40 h of storage, 96.8 ± 1.0% (n = 4) of the initial value was detected. Within a day of storage, no significant loss was detected.

The peaks corresponding to PTC ε-(γ-glutamyl)lysine (Figure 4b) and authentic PTC ε-(γ-glutamyl)lysine were collected and converted to PTH form followed by hydrolysis with HCl at 150 °C. The hydrolysates were resolved by the reversed-phase HPLC. In both cases, two major peaks were seen; the retention times coincided with the hydrolyzed PTH glutamic acid and lysine that were prepared under the same condition (Figure 5). Preliminary experiment of the hydrolysis of PTH ε-(γ-glutamyl)lysine showed that the peak area of these two derivatives increased linearly depending on the hydrolysis time until 10 min. After 20 min of hydrolysis, the peak area decreased slightly (data not shown). Then we chose 15 min for hydrolysis. This is direct evidence demonstrating the presence of ε-(γ-glutamyl)lysine cross-link in sardine meat gel, which may be formed by Ca^{2+}-dependent endogenous transglutaminase. The content of ε-(γ-glutamyl)lysine cross-link in sardine gel was 18.6 ± 1.8 mol/g of protein (n = 3).

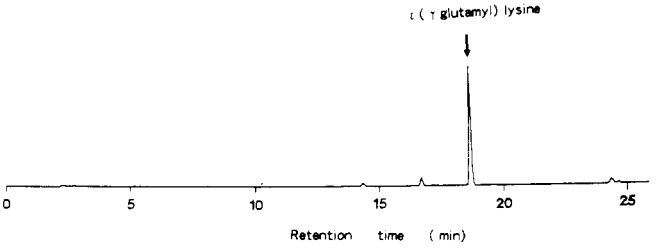

Figure 3. Chromatogram of the proteolytic enzyme-treated ε-(γ-glutamyl)lysine.

DISCUSSION

For the past decade, sensitive methods for the determination of ε-(γ-glutamyl)lysine cross-link in proteins using exhaustive proteolytic digestion and HPLC separation have been developed (Griffin et al., 1984; Beninati et al., 1988; Tarcsa and Fesus, 1990). In these studies, separation of other protein-constituting amino acids has not been optimized and data on the recovery of amino acids in proteolytic digest has not been described. In the present study, simultaneous determination of ε-(γ-glutamyl)lysine and protein-constituting amino acids has been achieved. This method has facilitated the monitoring of the progress of proteolytic digestion in sample proteins. In some proteolytic digestion conditions including the method reported previously (Griffin et al., 1984), many unknown peaks, which might be PTC peptides, appeared in chromatograms and recovery of glutamine was significantly low. Therefore, it was necessary to optimize the proteolytic condition to liberate amino acids from the protein of interest. In the present study, a proteolytic condition which allows sufficient liberation of amino acids, especially glutamine and lysine, from sardine meat sol has been established.

The concentration of ε-(γ-glutamyl)lysine cross-links is usually very low compared to those of other amino acids in protein. In addition, there have sometimes been occurrences of incomplete resolution of ε-(γ-glutamyl)-lysine derivatives from other peaks and the appearance of unknown peaks (Griffin et al., 1984; Nonaka et al., 1989; Tarcsa and Fesus, 1990; Kimura et al., 1991). As a result, additional identifications of ε-(γ-glutamyl)lysine have been performed by adding authentic ε-(γ-glutamyl)lysine (Griffin et al., 1984) and by using γ-glutamylamine cyclotransferase (Tarcsa and Fesus, 1990). In this study, a direct identification of ε-(γ-glutamyl)lysine cross-link in sardine meat gel was achieved by conversion to stable PTH ε-(γ-glutamyl)lysine followed by HCl hydrolysis. Conversion to the PTH form from PTC peptides resolved by HPLC may be a useful method to identify and confirm unknown peptide peaks, if present, in proteolytic digest.

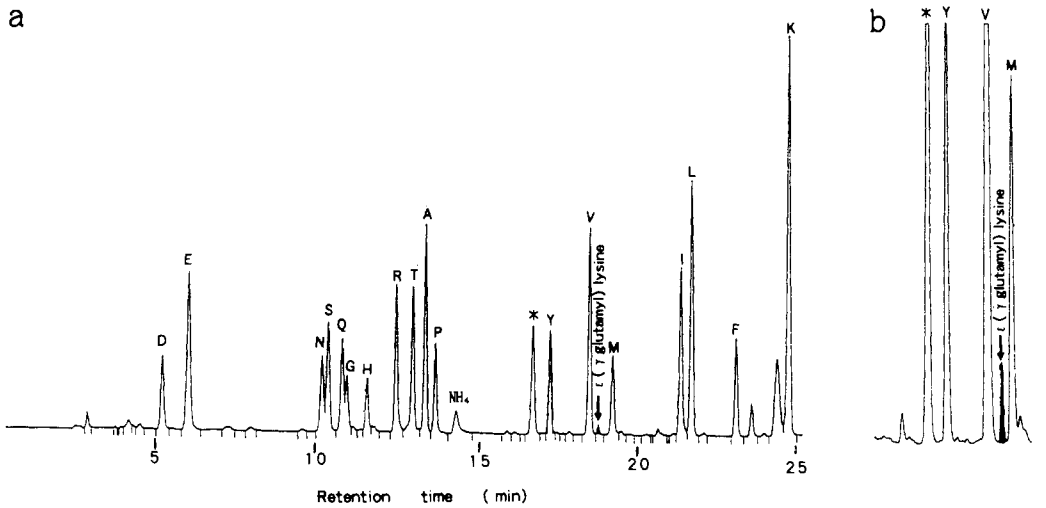

Figure 4. Chromatograms of proteolytic digest of sardine meat gel which was formed by incubation with $CaCl_2$. (a) Ten microliters of proteolytic digest was derived and diluted to 100 μL, and then 20 μL was injected. (b) Three hundred microliters was derived and diluted to 100 μL, and then 20 μL was injected. The peak corresponding to ε-(γ-glutamyl)lysine was collected and used for conversion to PTH form. Refer to the legend of Figure 1 for abbreviations.

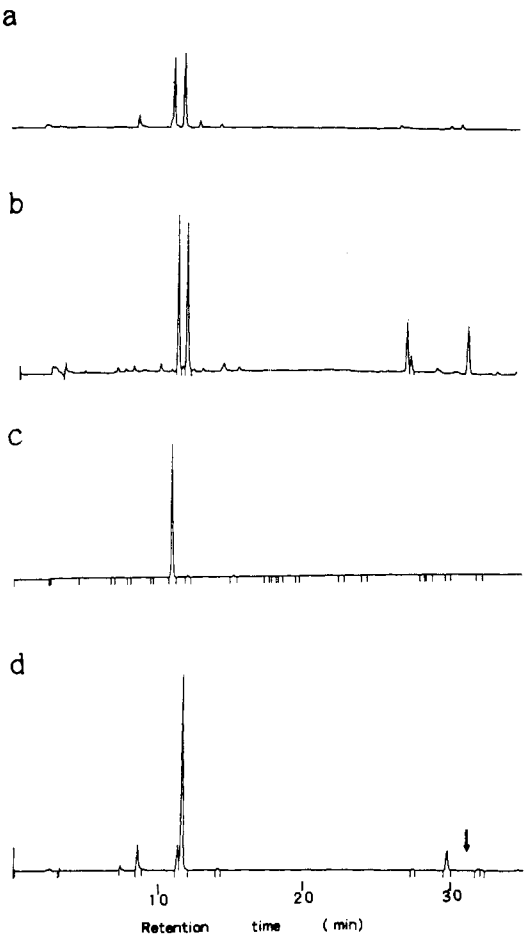

Figure 5. Chromatograms of HCl hydrolysate of PTH ε-(γ-glutamyl)lysine, glutamic acid, and lysine. (a) ε-(γ-Glutamyl)-lysine fraction in proteolytic digest of sardine meat gel (Figure 4b); (b) authentic ε-(γ-glutamyl)lysine; (c) glutamic acid; (d) lysine. Glutamic acid and lysine were also converted to PTH form followed by HCl hydrolysis. The arrow shows the elution position of PTH–ε-PTC lysine, which did not suffer from HCl hydrolysis.

This is one of the advantages of the PITC-based method over the OPA-based one.

Tarcsa and Fesus (1990) reported that a two-step preliminary chromatography purification was unavoidable in determining ε-(γ-glutamyl)lysine by use of PITC derivatization. In this study, we were able to determine ε-(γ-glutamyl)lysine in sardine meat gel without any interferences by a single ultrafiltration step to remove the proteolytic enzymes. If a sample contained impurities such as pigments or additives, the sample could be cleaned up before derivatization by using the Sep-Pak C_{18} cartridge (Waters). We have confirmed that no ε-(γ-glutamyl)-lysine before the derivatization was bound to a Sep-Pak C_{18} cartridge equilibrated with 0.1 M HCl. After the first effluent of 1.5 mL is eliminated, the remaining effluent from the cartridge can be used for the determination of ε-(γ-glutamyl)lysine. These results show that derivatization with PITC followed by HPLC separation as described in this paper is a reliable and sensitive means for the identification and determination of ε-(γ-glutamyl)-lysine cross-links in food proteins. Further study of the relationship between the rheological properties of food and a number of ε-(γ-glutamyl)lysines formed by endogenous and/or exogenous transglutaminases is now in progress.

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