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Rapid, single-step procedure for the identification of transglutaminase-mediated isopeptide crosslinks in amino acid digests

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Abstract

Tissue transglutaminase (tTG) is a calcium-activated enzyme which can covalently crosslink the ϵ-amino group of a peptide-bound lysine into the γ-carboxamide group of a peptide-bound glutamine, forming a ϵ-(γ-glutamyl)lysine isopeptide bond. We have developed a sensitive, single-step method for the isolation and detection of tTG-mediated isopeptide bonds from purified proteins and tissue homogenates. This method offers significantly improved resolution over current techniques, and obviates the need for multi-column systems or costly fluorescence monitors. By using enzymatic proteolysis, derivatization with phenylisothiocyanate, and a simple elution gradient for HPLC, we were able to determine the frequency of crosslinks in purified fibrin (1.7 mol of isodipeptide per mol of fibrin), crosslinked τ proteins (0.75 mol of isodipeptide per mol of τ), and whole-tissue liver homogenates (0.5 nmol of isodipeptide per mg of total protein).

Keywords: Enzymes; Transglutaminase; Amino acid digests; Isopeptide crosslinks

1. Introduction

The transglutaminases (TG) are a family of calcium-activated, thiol-dependent enzymes that can crosslink substrate proteins into high-molecular weight complexes that are insoluble and protease-resistant [1]. Six distinct TG genes have been identified in mammals, including blood coagulation factor XIIIa, epidermal, keratinocyte, prostate, and tissue type TG [2], and a newly discovered TGX, which was found in human keratinocytes using PCR [3]. Various transglutaminases are involved in skin, hair and nail cornification, hepatic fibrogenesis, fibrin crosslinking during clot formation, and the formation of cataracts in the eye lens (for a review, see Refs. [2,4,5]).

The binding of calcium by TG exposes an active-site cysteine residue that can react with the γ-carboxamide of a substrate glutamine, forming an intermediate γ-glutamyl thioester and releasing ammonia [1,4,6]. The acyl-enzyme intermediate then reacts with a nucleophilic primary amine, resulting in an incorporation of the amine-containing 'donor' into the substrate glutamyl 'acceptor.' If the primary

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amine is donated by the ε-amino group of a peptide-bound lysine, the result is a crosslinking of the two proteins via the formation of a ε-(γ-glutamyl)lysine isopeptide bond. Since this isopeptide bond is a unique product of TG activity, the detection of such bonds is a key step in determining whether a putative substrate can be crosslinked by the enzyme.

As the TG-mediated crosslinking of proteins produces a covalent modification between the side chains of glutamine and lysine, the resulting ε-(γ-glutamyl)lysine isopeptide bonds are not susceptible to cleavage by proteases that hydrolyze ordinary peptide bonds. Thus, isolation and detection of ε-(γ-glutamyl)lysine is generally accomplished using exhaustive proteolysis of the crosslinked proteins, followed by chromatographic purification of the isodipeptide [7]. Several chromatographic methods have been used to isolate the isodipeptide, including paper, thin-layer, and ion-exchange chromatography [7]. However, as the TG-mediated crosslink generally occurs in a low stoichiometry, the most reliable method of isolation involves RP-HPLC [7,8].

Because the single amino acids generated by exhaustive proteolysis cannot be easily detected using a spectrophotometer, derivatization of the amino acids prior to detection is necessary. Pre-column derivatization with *o*-phthalaldehyde (OPA) has proved to be useful for such detection [9,10]; however, this protocol requires the presence of a fluorescence monitor, and OPA does not react with proline and hydroxyproline residues [11]. Furthermore, the fluorescent products are relatively unstable, and present difficulties in quantification due to the sensitivity to quenchers [12]. To overcome these problems, an alternate derivatization technique using phenylisothiocyanate (PITC) has also been developed [11,12]. This method allows for rapid and reproducible detection of low levels of amino acids, and the phenylthiocarbamyl (PTC) derivatives are relatively stable at 4°C [13].

Derivatization of amino acids with PITC has been successfully employed in the detection of ε-(γ-glutamyl)lysine bonds [8]. Unfortunately, the elution conditions used in these studies did not achieve optimal separation of derivatized enzyme-digested amino acids in the vicinity of the isodipeptide. The ε-(γ-glutamyl)lysine isodipeptide eluted as a very weak shoulder on a large neighboring amino acid peak [8,14]. The method also requires a preliminary purification step using a cation exchanger resin, as well as HPLC separation on a silica column prior to derivatization.

In the present study, PITC-derivatized amino acid samples are separated using RP-HPLC with a simple one-step elution gradient which results in excellent separation of the ε-(γ-glutamyl)lysine isodipeptide from adjacent amino acids. No prior purification steps are necessary, and the entire process is completed in 60 min, including wash steps before and after the elution. Using this new method, the ε-(γ-glutamyl)lysine isodipeptide was isolated from purified human fibrin, and the extent of detected crosslinking was similar to that determined previously [15]. The extent of isodipeptide formation was also determined after the in vitro incubation of tissue type TG (tTG) with the microtubule-associated protein τ, as this reaction may be of potential relevance to Alzheimer's disease [16]. Isodipeptide bonds were also isolated from the crude liver homogenate of a 2-year-old rat, indicating that the present method is useful in detecting the presence of isodipeptide in physiological samples as well.

2. Experimental

2.1. Materials

Guinea pig liver tTG, fibrin (human plasma, washed), bovine serum albumin (BSA), PITC, triethylamine (TEA), sodium acetate, methanol, acetonitrile, and individual amino acids were purchased from Sigma–Aldrich (St. Louis, MO, USA). Amino acid standard-H and the BCA Protein Determination Kit were purchased from Pierce (Rockford, IL, USA). The isodipeptide ε-(γ-glutamyl)lysine, as well as glutamine-ε-lysine and glu–lys dipeptide, were purchased from Bachem Bioscience (King of Prussia, PA, USA). All proteases were purchased from Boehringer Mannheim (Indianapolis, IN, USA) except leucine aminopeptidase (Sigma–Aldrich) and carboxypeptidase A (Worthington, Lakewood, NJ, USA). Tau from bovine brains was purified as described previously [17], and the recombinant human τ isoform T4L [18] was expressed and cloned

as described by Fleming et al. [19]. Liver from a 2-year-old Sprague–Dawley rat, which had been used for hepatotoxin studies, was kindly donated by Dr. Michael Wyss of the University of Alabama at Birmingham.

2.2. Equipment

Peptide digests and amino acid standards were dried and derivatized using the Waters (Milford, MA, USA) Pico-Tag System. Separation was achieved using a DX300 HPLC system, with a GPM gradient pump module and a VDM-II variable wavelength detector (Dionex, Sunnyvale, CA, USA). The Vydac (Hesperia, CA, USA) reversed-phase C_{18} column (218TP-5405, 5 μm, 4.6×50 mm) was maintained at 30°C using a column heater (Dionex). Derivatized PTC-amino acids were detected at 254 nm.

2.3. Chromatographic conditions

Amino acid digests were separated using the program outlined in Table 1. Buffer 1 consisted of 70 m*M* ammonium acetate (pH 6.55), 2.5% acetonitrile, 0.2 μg/ml EDTA, and 0.05% triethylamine. Buffer 2 was 45% acetonitrile, 15% methanol in water. Buffers were degassed by sonication under vacuum for 1 min.

2.4. Standard solutions and calibration

Using the method of Heinrikson and Merdith [12], amino acid preparations were dried under vacuum and redissolved in 10–20 μl of a drying solution containing 1 *M* sodium acetate, methanol, and triethylamine in a ratio of 2:2:1. The samples were again dried under vacuum, then reconstituted in a freshly made derivatizing solution containing methanol, water, triethylamine, and PITC in a ratio of 7:1:1:1. Derivatization was performed at room temperature for 10 min, using occasional sonication and repeated vortexing to ensure complete resolubilization. The samples were dried as above, then redissolved in 200 μl of 7:2 water–acetonitrile. Aliquots (usually 5–20 μl) were injected into a reversed-phase C_{18} column, and separated using the program described in Table 1. Because of minor batch-to-batch eluent variations and changing elution properties due to settling of the eluents, slight variations in retention times (less than ±0.5 min) were occasionally noticed. In all experiments, amino acid standards and commercially available isodipeptide were used to calibrate the elution profile prior to data analysis.

2.5. Sample preparation

Bovine τ and the recombinant human τ isoform T4L were crosslinked by tTG as previously de-

Table 1
Elution conditions

Time (min)	Flow-rate (ml/min)	Buffer 1 (%)	Buffer 2 (%)	Comments
0.0	0.1	0	100	Wash
0.1	1.0	0	100	
2.5	1.0	0	100	
2.6	1.0	100	0	Equilibrate
10.0	1.0	100	0	Reset absorbance
10.1	1.0	100	0	Inject
19.0	1.0	78	22	
34.0	1.0	68	32	
41.0	1.0	56	44	
42.5	1.0	0	100	Wash
50.0	1.4	0	100	
54.0	1.4	0	100	
60.0	1.4	0	100	

scribed [16]. Crosslinked bovine τ (5 mg/ml), fibrin (10 mg/ml), BSA (10 mg/ml), or crosslinked T4L (1 mg/ml) were subjected to exhaustive enzymatic proteolysis. Several different combinations of proteases have been successfully used by this laboratory, and the results are consistent, provided that complete proteolysis into amino acids occurs. The protocol for proteolyzing crosslinked bovine τ was as follows:

Bovine τ (1 mg) which had been crosslinked by tTG was incubated in 200 μl of a buffer containing 10 m*M* Tris (pH 7.0) and a small crystal of thymol to inhibit bacterial growth. Pronase was added (1:20), and the solution was incubated at 37°C for 24 h. The pH of the buffer was adjusted to 8.0, and proteinase K was added (1:20) and incubated for 24 h at 50°C. Magnesium chloride was added to a concentration of 5 m*M*, and the sample was incubated with leucine aminopeptidase (1:25) for 24 h at 37°C. Carboxypeptidases A and Y were added (1:25), and incubated for 1 h at 37°C. As a control for proteolysis of the proteases themselves, a parallel sample was prepared which contained the proteases alone. Between each addition of protease, the samples were boiled for 5 min to inactivate the previous protease, then sonicated. The final samples were diluted to the same volume to compensate for evaporation, and the samples were centrifuged through 10-kDa filters (Waters Corporation). An aliquot of each sample (30 μl) was combined with 2 μl of 500 m*M* NaCl and 30 nmol of norleucine, an internal standard. The samples were derivatized and isodipeptide content was determined as described above.

2.6. Preparation of physiological tissue samples

As apoptotic hepatocytes are known to contain substantial amounts of tTG-mediated ε-(γ-glutamyl)lysine isodipeptide bonds [20], the liver of a 2-year-old Sprague–Dawley rat which had been used for hepatotoxin studies was utilized as a physiological tissue sample in our experiments. The liver was removed, weighed, and homogenized as described by Fesus and Arato [21]. As a positive control, a portion of the homogenate (0.325 g) was incubated with tTG (1:1000) in the presence of 2 m*M* exogenous $CaCl_2$ for 10 min at 37°C. Tween-20 was added to 0.1%, and the samples were sonicated. Protein concentrations were determined using the BCA method (Pierce). Total protein (6.0 mg each) was precipitated at 4°C for 30 min using 10% trichloroacetic acid (TCA) in a volume of 400 μl. The pellets were washed three times with 600 μl of diethyl ether, and dried overnight in a fume hood. As a further positive control, commercially available isodipeptide (2 nmol/mg protein) was exogenously added to one of the samples which had not been incubated with tTG. Each of the samples was exhaustively proteolyzed in 200 μl of a buffer containing 10 m*M* Tris (pH 8.0), 0.1% SDS, and a small crystal of thymol. These samples, as well as a blank sample to control for the proteolysis of the proteases themselves, were incubated at 37°C (unless otherwise indicated) with the following proteases: pronase (1:50, 24 h), proteinase K (1:50, 24 h, 50°C), subtilisin (1:100, 24 h), proteinase K again (1:100, 24 h, 50°C), leucine aminopeptidase (1:200, 24 h, with 5 m*M* $MgCl_2$), carboxypeptidase Y (1:100, 2.5 h), carboxypeptidase A (1:100, 1.5 h), and carboxypeptidase B (1:500, 6.5 h). Samples were concentrated using a speed-vac centrifuge, rediluted to 200 μl, and processed as above, except that the derivatization time was extended to 20 min.

2.7. Acid hydrolysis

Acid hydrolysis of amino acid digests was performed by incubating the samples in the presence or absence of 6 *M* HCl, at 110°C for 18 h. The samples were blanketed with nitrogen and sealed in glass conical vials prior to hydrolysis.

3. Results and discussion

Mixtures containing the acid-hydrolyzed amino acid standards were supplemented with asparagine, cysteine, glutamine, tryptophan, and norleucine internal standard, with and without the addition of ε-(γ-glutamyl)lysine isodipeptide. These samples were derivatized with PITC and separated using the HPLC elution program indicated in Table 1. A typical chromatographic pattern of these samples is shown in Fig. 1. The retention times of the amino acids were verified by derivatizing and applying them

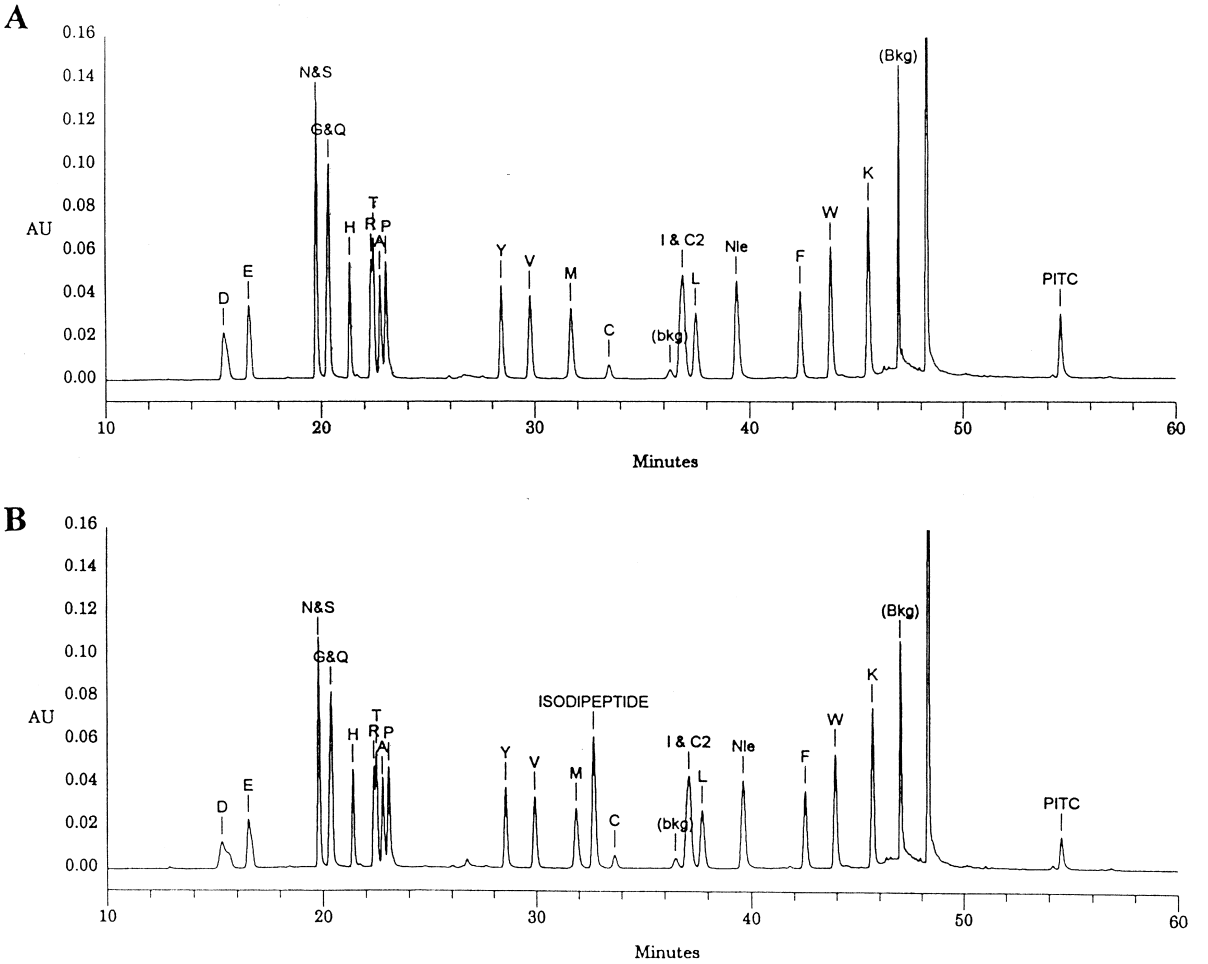

Fig. 1. Typical chromatographic pattern of PITC-derivatized amino acid standards, in the absence (A) or presence (B) of purified PITC-derivatized ϵ-(γ-glutamyl)lysine isodipeptide. The isodipeptide peak (32.86±0.16 min) is well-resolved and elutes between methionine (31.50±0.25 min) and cysteine (33.83±0.11 min). The elution times (mean±SEM) are given in parentheses and were obtained from a series of 10 consecutive runs. Although slight variations in elution times can occur in some cases, the elution position of ϵ-(γ-glutamyl)lysine relative to methionine and cysteine remains constant.

individually to the HPLC. The isodipeptide elutes as a sharp, well-resolved peak between methionine and cysteine. As cysteine is generally converted to cystine during PITC derivatization, the peak due to this amino acid is minimal. Under the chromatographic conditions used, ϵ-(γ-glutamyl)lysine isodipeptide elutes at 32.86 min (Fig. 1) and is clearly separated from other similar molecules, such as glutamine-ϵ-lysine (34.78 min) and glu–lys dipeptide (38.08 min) (data not shown). A concentration curve of isodipeptide was generated using isodipeptide concentrations between 15 and 250 pmol, and is represented by the equation $y=92.73x-322$ (correlation coefficient of 0.999). The curve is linear between 15 and 250 pmol of isodipeptide, and the limit of detection for purified isodipeptide is 12 pmol. The inter-day variations in peak height are negligible, although peak elution times can fluctuate slightly due to buffer conditions and temperature.

To verify that the HPLC method is useful for the isolation of physiological isodipeptide, washed human fibrin, which is known to contain ϵ-(γ-

glutamyl)lysine isopeptide bonds [15], and BSA, which does not contain isodipeptide bonds [16], were enzymatically proteolyzed into amino acids. After derivatization and HPLC separation, the amino acid digest of fibrin was found to contain a significant concentration of isodipeptide, whereas the digests of BSA and the proteases alone did not (Fig. 2). Based upon the calibration curve, 1 nmol of fibrin was determined to contain 1.7±0.3 nmol of isodipeptide. This result is comparable to a previously published result of 1–1.4 nmol of isodipeptide per nmol of fibrin [15].

The microtubule-associated protein τ has also been shown to be a substrate of tTG, and it has been proposed that tTG crosslinking may be involved in the formation of insoluble neurofibrillary tangles in Alzheimer's disease [16,22]. To determine the stoichiometry of isodipeptide formation by tTG in τ, soluble bovine τ or human recombinant τ isoform T4L [18] were incubated in vitro with or without calcium and tTG, and the resulting proteins were subjected to proteolysis and analyzed. Crosslinked bovine τ (Fig. 3, upper trace) and the crosslinked human τ isoform (data not shown) were found to contain comparable amounts of the isodipeptide, whereas no isodipeptide was detected in the τ sample which had not been incubated with tTG (Fig. 3, lower trace). The frequency of isodipeptide formation in crosslinked τ was determined to be 0.75±0.12 mol of isodipeptide per mol of τ in our experiments. This value is significantly lower than the number of glutamines within τ that can be modified by tTG, as determined by polyamine incorporation assays [16,23]. Considering that crosslinking is a highly specific occurrence, not all potential substrate glutamines and lysines would be expected to participate. As polypeptide regions of substrate proteins associate, only substrate glutamines that are closely apposed to substrate lysines will become crosslinked. The data from these

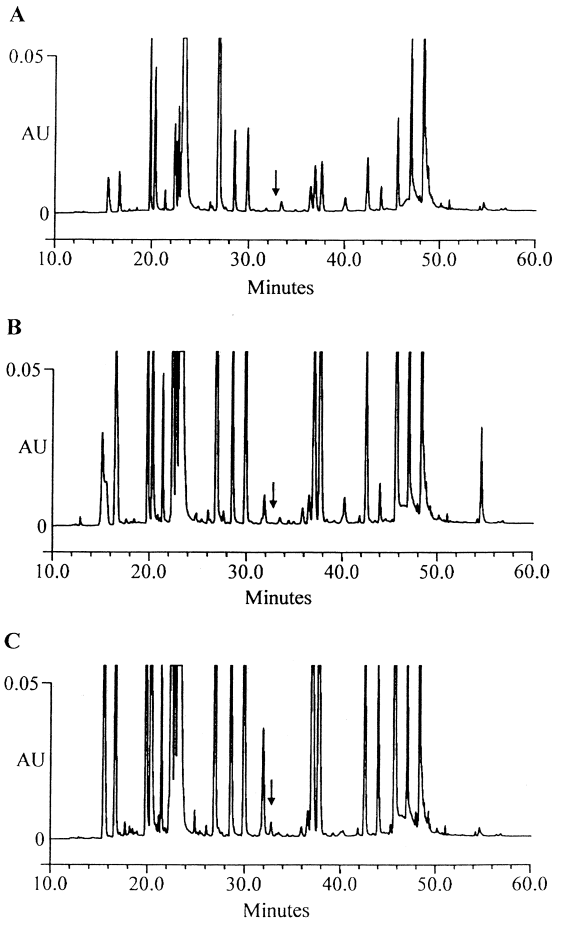

Fig. 2. Chromatographic patterns of the PITC-derivatized enzymatic digests of BSA (B) and fibrin (C) and of the proteases alone (A). Aliquots corresponding to approximately 17 μg of total protein were injected into the HPLC system. A peak corresponding to derivatized ε-(γ-glutamyl)lysine isodipeptide can be seen in the elution profile of fibrin, but not in the profile of BSA or the protease control (arrows).

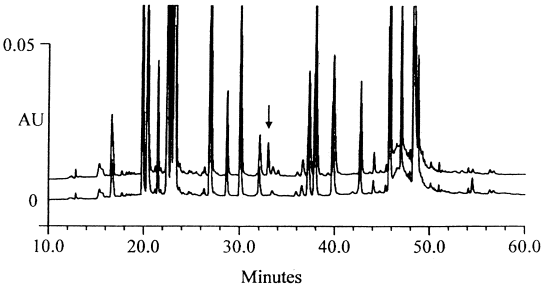

Fig. 3. Chromatographic patterns of the PITC-derivatized enzymatic digests of tissue transglutaminase-crosslinked (upper trace) and noncrosslinked (lower trace) bovine τ. Aliquots corresponding to approximately 7 μg of total protein were injected into the HPLC system. The ε-(γ-glutamyl)lysine isopeptide bond (arrow) is present in crosslinked bovine τ, but not in τ which has not been crosslinked.

experiments indicate that τ is crosslinked at a substoichiometric ratio, and it is clear that not every site within τ that can be modified by tTG is actually crosslinked by the enzyme. It is also likely that the kinetics of τ association and other reaction conditions may have inhibited complete crosslinking by tTG.

A necessary requirement for isodipeptide isolation is the ability to identify them in physiological tissue samples. Since apoptotic hepatocytes are known to contain substantial amounts of ε-(γ-glutamyl)lysine isodipeptide bonds [20], the liver of a 2-year-old Sprague–Dawley rat that had been used for hepatotoxin studies was utilized as a physiological tissue sample in our experiments. As a positive control, a portion of the liver homogenate (0.325 g) was incubated in the presence of calcium and tTG, to further enhance the amount of isodipeptide in the sample. Total protein (6 mg) was acid-precipitated from each homogenate, and as a further positive control commercially available isodipeptide was exogenously added to one of the samples which had not been incubated with tTG. The samples were then subjected to exhaustive enzymatic proteolysis, derivatized, and analyzed.

As seen in Fig. 4A, a significant amount of isodipeptide can be detected in the homogenate of aged rat liver. According to the isodipeptide calibration curve, the concentration of isodipeptide is approximately 0.5 nmol of isodipeptide per mg of total protein. The homogenate which had been incubated with tTG (Fig. 4B) was found to contain 66% more isodipeptide than the non-crosslinked homogenate, and the homogenate which had been spiked with isodipeptide prior to proteolysis (Fig. 4C) was found to contain over 350% more crosslink.

Because ε-(γ-glutamyl)lysine isodipeptide bonds are acid labile, the amino acid digest from aged rat liver homogenate was incubated in the presence or absence of 6 *M* HCl, at 110°C for 18 h. Incubation in the absence of HCl did not cause any changes in the chromatographic pattern (Fig. 5A). The treatment of the amino acid digest with HCl (Fig. 5B) caused a disappearance of the isodipeptide peak. Unfortunately, incubation with acid also caused a slight change in the elution of certain peaks, including a large peak that elutes immediately before the isodipeptide. This large peak does not interfere with the detection of isodipeptide, however, as the addition of 75 pmol of isodipeptide prior to injection results in a quantitative recovery of the missing peak (Fig. 5C).

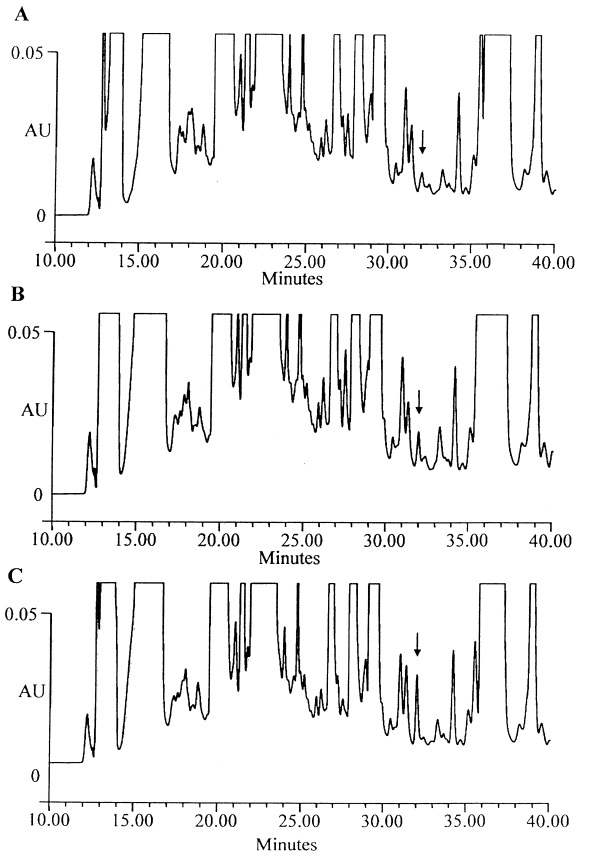

Fig. 4. Chromatographic patterns of the PITC-derivatized enzymatic digests of a liver homogenate from an aged rat. Aliquots corresponding to approximately 170 μg of total protein were injected into the HPLC system. A significant amount of ε-(γ-glutamyl)lysine isodipeptide can be detected in the aged rat liver (A). When rat liver homogenate was incubated in the presence of tissue transglutaminase and calcium prior to digestion. the amount of isodipeptide detected increased by 66% (B). As a control, purified isodipeptide (2 nmol/mg protein) was added to the noncrosslinked liver homogenate prior to digestion (C). Arrows indicate the position of ε-(γ-glutamyl)lysine.

Thus, we have developed a rapid, sensitive technique that is useful for the detection of tTG-catalyzed ε-(γ-glutamyl)lysine isodipeptide bonds from enzyme-digested proteins and tissue homogenates. Because the isodipeptide is clearly separated from other homogenate components, this technique offers

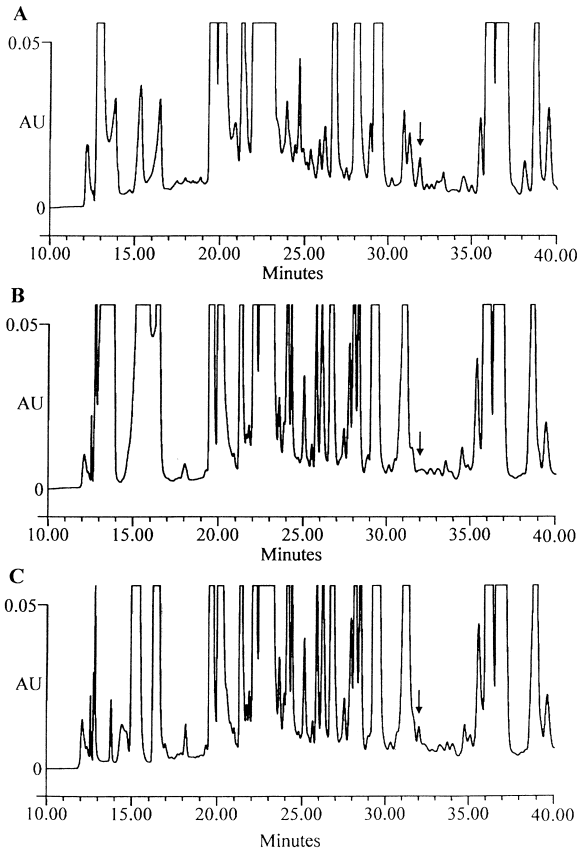

Fig. 5. Chromatographic patterns verify that the ε-(γ-glutamyl)lysine isodipeptide is acid labile. Prior to derivatization, amino acid digests of rat liver homogenate were acid hydrolyzed in the absence or presence of 6 *M* HCl. Homogenate incubated in the absence of HCl exhibits an elution profile identical to that shown in Fig. 4A (A). Incubation of the homogenate with HCl abolished the peak corresponding to ε-(γ-glutamyl)lysine (B). Addition of commercially available ε-(γ-glutamyl)lysine to the acid-hydrolyzed homogenate yielded quantitative recovery of the isodipeptide peak (C). Arrows indicate the position of ε-(γ-glutamyl)lysine.

significantly improved sensitivity over the established PITC derivatization method [8,14]. The improved resolution obviates the need for prior purification steps, and results in a simpler, more rapid assay. Such a method should enable new laboratories to undertake the analysis of isodipeptide bonds without the need for costly fluorescence detectors or multiple column systems.

Acknowledgements

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