Quantitative Analysis of Pyroglutamic Acid in Peptides

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A simplified and rapid procedure for the determination of pyroglutamic acid in peptides was developed. The method involves the enzymatic cleavage of an N-terminal pyroglutamate residue using a thermostable pyroglutamate aminopeptidase and isocratic HPLC separation of the resulting enzymatic hydrolysate using a column switching technique. Pyroglutamate aminopeptidase from a thermophilic archaebacteria, *Pyrococcus furiosus*, cleaves N-terminal pyroglutamic acid residue independent of the molecular weight of the substrate. It cleaves more than 85% of pyroglutamate from peptides whose molecular weight ranges from 362.4 to 4599.4 Da. Thus, a new method is presented that quantitatively estimates N-terminal pyroglutamic acid residue in peptides.

Keywords: *Pyroglutamic acid; peptide; pyroglutamate aminopeptidase; 5-oxoprolyl peptidase*

INTRODUCTION

The amino acid composition of proteins or peptides used in food or feed is routinely assessed by the AOAC method (AOAC, 1997). It involves acid hydrolysis and HPLC analysis. The acid hydrolysis converts glutamine (Gln) and pyroglutamic acid (pGlu) to glutamic acid (Glu). Thus, the three amino acids are analyzed as Glu and collectively expressed as Glx.

Gln, which is classically categorized as a nonessential amino acid, has been shown to have a unique role in the immune system (Ardawi and Newsholme, 1985) and the gastroenteral tract (Souba, 1991). Therefore, it is regarded as a conditionally essential amino acid (Lacey and Wilmore, 1990). This has encouraged the development of a method to assess glutamyl residue content in natural proteins and peptides (Kuhn et al., 1996a) and in enteral products (Kuhn et al., 1996b).

Pyroglutamic acid (pGlu) residue exists solely at the N-terminus of peptides or proteins. Although some hormones or naturally occurring peptides are known to have pGlu, a pGlu-containing protein has only one pGlu at the N-terminal position; thus, the amount was expected to be an insignificant level compared with other naturally occurring amino acids in food proteins. Consequently, pGlu has been virtually neglected in the nutritional assessment of the amino acid content. Recently, the occurrence of pyroglutamyl peptides in an industrially prepared peptide was demonstrated (Sato et al., 1998). Since the amino group and amide group of Gln easily condense to form pGlu under aqueous conditions, industrial partial hydrolysis of protein may produce a significant amount of pyroglutamyl peptide when Gln residue is exposed at the N-terminal position.

The Glx content of proteins is normally between 10% and 20% except in unique proteins such as wheat gluten (40%). A protein molecule usually has one N-terminal residue, which is negligible when considering the entire amino acid composition. Therefore, the Gln and Glu content has been sufficient for assessment of Glx in protein. However, partially hydrolyzed proteins are widely used in processing food, and if they have a significant amounts of pyroglutamyl peptides, the situation changes.

Mammals are known to have pyroglutamate aminopeptidase [EC 3.4.19.3], which releases pGlu from pyroglutamyl peptide, and 5-oxoprolinase [EC 3.5.2.9], which opens the pyrrolidone ring of pGlu to form Glu in an ATP-dependent manner. Therefore, the metabolic fate of pGlu in peptide form is considered to be the same as that of Glu in peptide, but the nutritional value still needs to be elucidated. The pGlu content of a peptide can be analyzed by sequence analysis. However, analysis of industrially prepared, complex peptide mixtures is not feasible with those methods. Therefore, a method is required to estimate pGlu in peptide mixtures.

MATERIALS AND METHODS

Reagents. Pyroglutamic acid, 2-mercaptoethanol, ethylenediaminetetraacetic acid, TRIZMA base (Sigma-Aldrich Japan, Tokyo, Japan), L-glutamine (Kanto Chemical, Tokyo, Japan), monosodium L-glutamate (Wako Pure Chemical Industries, Tokyo, Japan), perchloric acid 70% (Sigma-Aldrich Japan, Tokyo, Japan), and 5-sulfosalicylic acid (Tokyo Kasei, Tokyo, Japan) were of analytical or biochemical grade. Ultrapure water was prepared by Milli-Q (Millipore Japan, Tokyo, Japan).

Peptides. Thyrotropin releasing hormone (TRH (free base)), substance P [$pGlu^6$, Pro^9]-fragment 6-11, luteinizing hormone releasing hormone (LH-RH (human)), bombesin, gastrin I (human), and sauvagine were purchased from Sigma-Aldrich Japan (Tokyo, Japan). All these peptides have pyroglutamic residues at the N-terminus and molecular weights ranging from 362.4 to 4599.4 Da (Table 1). Peptide solutions were prepared at concentrations between 0.5 and 1.0 nmol/μL according to the manufacturer. Aliquots of the solutions were hydrolyzed with HCl, and amino acid concentrations were analyzed by the Amino Acid Analyzer L-8500 (Hitachi, Tokyo, Japan). The actual concentrations of the peptide solutions were

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Table 1. Recovery of pGlu (Mean of Duplicate Determinations) from Bioactive Peptides with pGlu Residue at the N-Terminal Position[a]

no.	peptide	2nd residue	AA residue	MW (Da)	yield of pGlu (%)
1	TRH (free base)	His	3	362.4	94.1
2	substance P [pGlu^6, Pro^9]-Fragment 6−11	Phe	6	764.0	97.7
3	LH−RH (human)	His	10	1182.3	86.5
4	bombesin	Gln	14	1619.9	93.8
5	gastrin I (human)	Gly	17	2098.2	99.2
6	sauvagine	Gly	40	4599.4	103.1

[a] Data are presented in comparison with the theoretical value.

calculated from the glutamate concentration in the aliquots (means of two separate analyses). All peptide solutions were stored at −80 °C.

Industrially prepared wheat gluten hydrolysates were prepared by the procedure described in our previous report (Sato et al., 1998); a low molecular weight hydrolysate was prepared following this procedure, and a high molecular weight hydrolysate was prepared by modifying the enzymatic reaction time.

Pyroglutamate Aminopeptidase. Pyroglutamate aminopeptidase from *Bacillus amyloliquefaciens* (Sigma-Aldrich Japan, Tokyo, Japan) and *Pfu* pyroglutamate aminopeptidase (Takara Biomedicals, Shiga, Japan) were used in the analyses. Enzymes were dissolved and diluted with reaction buffer (10 mM 2-mercaptoethanol, 5 mM ethylenediaminetetraacetic acid, 50 mM Tris·HCl (pH7.5)) and stored at 4 °C. The concentrations of the *Bacillus* and *Pfu* enzymes were 1.0 unit/μL and 0.05 mU/μL, respectively, according to the manufacturer's unit definitions.

Enzymatic Cleavage of the N-Terminal Pyroglutamate Residue. Not more than 100 nmol of peptide was dissolved in 90 μL of reaction buffer. Diluted enzyme (10 μL) was added to this peptide solution. The mixture was kept at the optimal temperature, for the *Bacillus* enzyme 37 °C and the *Pfu* enzyme 50 °C, for up to 120 min using TaKaRa PCR Thermal Cycler 480 (Takara Biomedicals, Shiga, Japan). After the reaction, the mixture was deproteinized as follows: 10 μL of 5-sulfosalicylic acid solution (1.5 g/100 mL H_2O) was added to the mixture which was mixed well, then filtered through a 0.22 μm membrane filter using Suprec-01 (Takara Biomedicals). The deproteinized sample was stored at −80 °C until HPLC analysis.

HPLC Apparatus and Procedure. The HPLC system is composed of two pumps, L-6000 (Hitachi, Tokyo, Japan) and 880-PU (Jasco, Tokyo, Japan), two model 7125 injectors (Rheodyne, Cotati, CA), two precolumns and an analysis column, a L-4200 UV detector (Hitachi), a model 556 column oven (Gasukuro Kogyo, Tokyo, Japan), and a Chromatocorder 12 integrator (Sic, Tokyo, Japan). The same strong anion-exchange resin was packed in the two guard columns, Shodex RSpack KC-LG (8 mm i.d. × 50 mm), and the analysis column, Shodex Rspack C-811 (8 mm i.d. × 500 mm) (Showa Denko, Tokyo, Japan). The guard columns were connected sequentially and used as precolumns.

Sequentially, a pump, an injector, the precolumns, the second injector, the analytical column, and the detector composed the main pass. The second pump was connected to the second injector. Each pump used perchloric acid (3 mM) as the solvent at a flow rate of 1 mL/min. All columns were kept at 40 °C in the column oven. The eluate from the analysis column was monitored at 220 nm.

Sample was injected into the first injector and carried into the precolumns. The eluate from 3.0 to 4.5 min was introduced into the analysis column using the second injector as a switch.

The pyroglutamate concentration of the sample was calculated by comparing the peak area with those of standard solutions of pyroglutamic acid (1.0−10 mM).

RESULTS AND DISCUSSION

HPLC Analysis of Pyroglutamic Acid in the Reaction Mixture. The wheat gluten hydrolysates were analyzed. When the deproteinized reaction mixture was introduced into the analysis column directly, pGlu was observed at a retention time of 18.6 min. Other peaks were observed up to 105 min. The precolumn separation of the pGlu fraction enabled us to observe pGlu at a retention time of 21−22 min and to shorten the analysis time to 26 min (Figure 1).

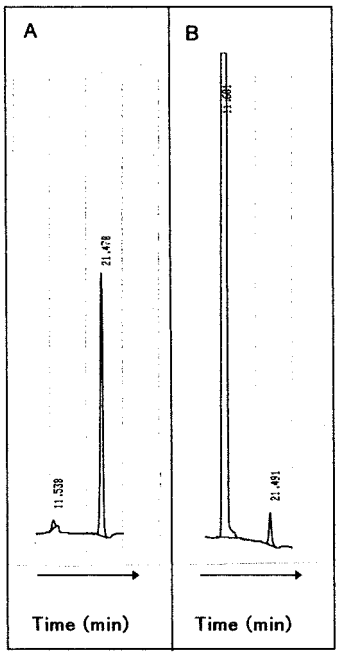

Figure 1. Chromatograms (8−26 min). Pyroglutamate is observed as a single peak at a retention time of 21.5 min in (A, left) pGlu standard (20 nmol) and (B, right) HPLC analysis of wheat gluten hydrolysate (low molecular weight type).

For the determination of pGlu, Nishimoto et al. (1979) proposed using HPLC separation with UV detection at 210 nm. Shih (1985), after examining some modification methods, concluded that the most effective way to detect pGlu was to measure its UV absorbance at 200 nm. Therefore, we chose an ion-exclusion column for the separation and detection with UV. Shodex RSpack C-811 was a column prepared for the organic acids analyses in which strong cation-exchange resin was packed. According to the manufacturer's instruction, we chose 3 mM perchloric acid as the solvent with UV detection at 220 nm.

Transformation of Pyroglutamate from Gln or Glu in the Reaction Buffer. Pyroglutamate formation from glutamine or glutamate in the reaction buffer was investigated. Glutamine or sodium glutamate (50 mM) in the reaction buffer was kept at a temperature ranging from 4 to 50 °C for up to 4.0 h or autoclaved (121, 15 or 30 min). As shown in Figure 2, glutamine is converted to pyroglutamate in a time and temperature-dependent manner. Under autoclave conditions, 78.5% and 93.7% conversion was observed at 15 and 30 min, respectively.

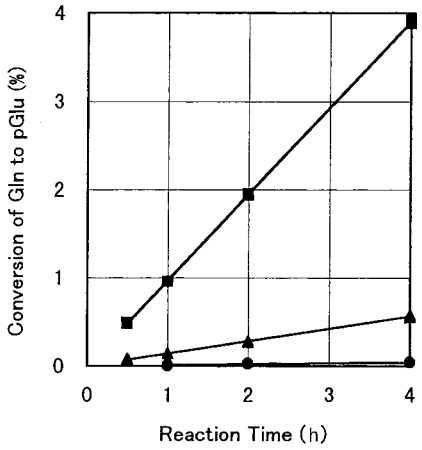

Figure 2. Conversion of Gln to pGlu in the reaction buffer. Glutamine (50 mM) in the reaction mixture was kept at 4 (●), 37 (▲), and 50 (■) °C for 0.5, 1, 2, 3, and 4 h. Duplicate data were obtained at every condition.

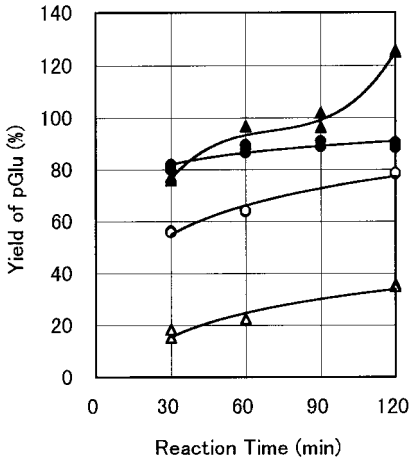

Figure 3. Pyroglutamate released from LH−RH (triangles) and bombesin (circles) by the *Bacillus* enzyme (open symbols) or the *Pfu* enzyme (closed symbols). Reaction temperatures were 37 °C for the *Bacillus* enzyme and 50 °C for the *Pfu* enzyme. Duplicate data were obtained at every condition.

In contrast, glutamate was much more stable in the buffer. Autoclaving for 30 min converted only 1.24% of glutamate to pyroglutamate.

Comparison of the Two Pyroglutamate Aminopeptidases. Pyroglutamate aminopeptidase or 5-oxoprolyl peptidase [EC 3.4.19.3] is widely distributed in bacteria (Tsuru et al., 1978), plants (Szewczuk and Kwiatkowska, 1970), and animal tissues (Taylor and Dixon, 1978). Some of the enzymes are commercially available and used for the selective deblocking of N-terminal pyroglutamic acids from peptides and proteins before Edman degradation in protein sequence analysis. Among them, the enzyme from *Bacillus amyloliquefaciens* (the *Bacillus* enzyme) is known to be stable (Tsuru et al., 1978) and have a high activity (Fujiwara et al., 1979). Recently, the enzyme from a thermophilic archaebacteria *Pyrococcus furiosus* (the *Pfu* enzyme) was produced by Takara Biomedicals. This is a thermostable enzyme with optimal temperature around 90 °C (Tsuanashima et al., 1998). According to the manufacturer's information, it is more stable and has a higher activity than the enzyme from pig liver (Takara, 1998). We compared the two enzymes, the *Bacillus* enzyme and the *Pfu* enzyme, using human LH−RH and bombesin as substrates.

For the reaction, excess enzyme was used according to the manufacturer's unit definitions. The reaction temperatures were 37 °C for the *Bacillus* enzyme and 50 °C for the *Pfu* enzyme. By 60 min, the *Pfu* enzymes released more than 85% of the pyroglutamate from both substrates. After incubation for 120 min, LH−RH released slightly more pyroglutamate (89.4%). Bombesin released more than the theoretical amount (125.2%). The *Bacillus* enzyme showed less activity than the *Pfu* enzyme at any point observed (Figure 3).

As shown in Figure 2, Gln is converted into pGlu in the reaction buffer in proportion to time and temperature. Therefore, if Gln appears at the N-terminal position after a reaction with pGlu aminopeptidase, the Gln could form pGlu by intramolecular cyclization to provide an additional substrate for the enzyme. This leads to overestimation of the pGlu content, as observed in Figure 3. The amino acid sequence of bombesin is pGlu-Gln-Arg−... from the N-terminal position. The reaction with *Pfu* enzyme for 120 min at 50 °C released more pGlu than its N-terminal amount, due to the secondary formation of pGlu. Thus, the possibility of this overestimation should be considered when choosing the time and temperature of the reaction.

Comparing the two enzymes, the *Pfu* enzyme showed higher activity at any point of the reaction than the *Bacillus* enzyme and almost 90% pGlu recovery from both substrates at 60 min, despite the difference in size. Therefore, the *Pfu* enzyme is more suitable for quantitative analysis of pGlu in peptides, although if the reaction time is longer than 60 min at 50 °C pGlu content could be overestimated.

Analysis of Bioactive Peptides. Pyroglutamate residue of six bioactive peptides was measured using the *Pfu* enzyme. The reaction was performed at 50 °C for 60 min. As shown in Table 1, more than 86% of pGlu was observed in any of the examined peptides.

An ideal method for estimating pGlu content in partially hydrolyzed protein would have the following features. First, content should be estimated independent of the molecular weight of the substrate, since partially hydrolyzed protein consists of variously sized peptides whose average molecular weight ranges from 300 to 5000 Da. The method, however, does not have to be applicable to bulky peptides or proteins. If a pGlu-containing peptide consists of 50 amino acid residues, the pGlu content is approximately 2% of the amino acid composition or 20% of the Glx at most, which could be negligible nutritionally. Secondly, pGlu should be equally estimated independent of the adjusting amino residues, since the hydrolysate is a mixture of various peptides.

The *Pfu* enzyme released more than 85% of the N-terminal pGlu from the peptides independent of their molecular weights (Table 1). This fulfills the first feature discussed above. As shown in Table 1, the *Pfu* enzyme quantitatively digested pGlu-containing peptide when the adjoining amino acid residue was histidine, phenylalanine, glutamine, and glycine. This enzyme cleaves pGlu from various pyroglutamyl peptides except when the adjoining amino acid is proline (Tsunashima, personal communication). Tsunashima et al. (1988), the originators of this enzyme, reported that this enzyme released pGlu which is bound to aspartic acid, leucine, glutamine, and alanine. Sato et al. used this enzyme in the sequence analysis of a pGlu-containing peptide whose second amino acid residue was asparagine, glutamine, isoleucine, phenylalanine, serine, tryp-

tophan, valine, threonine, and aspartic acid (data not shown). Thus, except the case when the second amino acid residue was proline, the *Pfu* enzyme was expected to liberate pGlu from pGlu-containing peptide universally. This almost satisfied the later condition discussed above. Therefore, the method should be applicable to estimate pGlu content in partially hydrolyzed peptides.

Analysis of Industrially Prepared Wheat Gluten Hydrolysate. Pyroglutamate content in the industrially prepared wheat gluten hydrolysates were examined. The average molecular weight of both hydrolysates was estimated by gel filtration: approximately 500 Da for the low molecular weight type and 3000 Da for the high molecular type (data not shown). The deproteinized enzymatic reaction mixture (30 μL) was introduced into the ion-exclusion column, and pGlu was observed as a single well-resolved peak (Figure 1). The pGlu contents of the low and high molecular weight type of the gluten hydrolysates were 0.486 and 0.133 mmol/g, respectively.

Using the same method, we have successfully analyzed 13 commercially available peptides (hydrolysate) of wheat gluten, corn gluten, soy protein, casein, and egg white (data not shown). Comparing the data, the pGlu content of 0.468 mmol/g was one of the highest values but 0.133 mmol/g was indifferent from some hydrolysates prepared from casein or corn protein.

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