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Isolation and Identification of Indigestible Pyroglutamyl Peptides in an Enzymatic Hydrolysate of Wheat Gluten Prepared on an Industrial Scale

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An enzymatic hydrolysate of wheat gluten was further digested in vitro with porcine pepsin and pancreatin to obtain an indigestible peptide. Indigestible pyroglutamyl peptide was isolated from the digest by strong cation-exchange, size-exclusion, and reversed-phase chromatographies. The pyroglutamyl peptide was digested with pyroglutamate aminopeptidase, and the digest was reacted with phenyl isothiocyanate. The resultant phenylthiocarbamyl (PTC) peptides were purified by reversed-phase HPLC by using binary gradient elution with ammonium acetate buffer, pH 6.0, and acetonitrile. The PTC peptides were analyzed with an automatic peptide sequencer on the basis of the Edman degradation method with a modified program. Some pyroglutamyl peptides were also analyzed by fast-atom bombardment ionization mass spectrometry without the pyroglutamate amino peptidase digestion. Consequently, pyroGlu-Asn-Pro-Gln, pyroGlu-Gln-Gln-Pro-Gln, pyroGlu-Gln-Pro-Gln, pyroGlu-Gln-Pro-Gly-Gln-Gly-Gln, pyroGlu-Gln, pyroGlu-Gln-Pro, pyroGlu-Ile-Pro-Gln, pyroGlu-Ile-Pro, pyroGlu-Gln-Pro-Leu, pyroGlu-Gln-Phe-Pro-Gln, pyroGlu-Ser-Phe-Pro-Gln, pyroGlu-Phe-Pro-Gln, and pyroGlu-Gln-Pro-Pro-Phe-Ser were identified.

KEYWORDS: Pyroglutamic acid; peptide; pyroglutamyl; indigestible; wheat gluten; pyroglutamate aminopeptidase; heat treatment; glutamine; HPLC; peptide sequence

INTRODUCTION

Supplementation of glutamine has been demonstrated to improve liver, kidney, and immune functions and also moderate muscle break down after strong exercise. Glutamine is now considered conditionally as an essential amino acid (*1*−*3*). However, free glutamine in aqueous solution is easily converted to pyroglutamic acid, which has no beneficial effect of glutamine. On the other hand, glutamine peptides, such as Ala-Gln and Gly-Gln, are stable in aqueous solution and have the beneficial effects of glutamine (*4*, *5*). On the basis of these findings, glutamine-rich peptides have been prepared from glutamine-rich protein, such as wheat gluten, on laboratory (*6*) and industry scales (*7*, *8*). Now, some beverages and clinical formulas containing such preparations are commercially available. In the previous studies, we found that such preparations contain significant amounts of pyroglutamyl peptide, which resists in vitro peptidase digestion (*8*, *9*). In some cases, the indigestible pyroglutamyl peptide accounts for more than 10% (w/w) of total peptides. However, knowledge about the structure of the pyroglutamyl peptide induced during the industrial preparation is limited (*10*). The objective of this study is to develop a method for purification and characterization of the indigestible pyroglutamyl peptide.

MATERIALS AND METHODS

Enzymes and Reagents. Porcine pepsin and pancreatin were obtained from Nacalai tesque (Kyoto, Japan). Pyroglutamate aminopeptidases from porcine kidney and *Pyrococcus furiosus* were obtained from TaKaRa (Kyoto, Japan). Trifluoroacetic acid (TFA), acetonitrile (HPLC grade), methanol (HPLC grade), and formic acid (HPLC grade)

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were obtained from Nacalai tesque. Triethylamine (TEA; sequence grade) was obtained from Pierce (Rockford, IL). Ethylenediaminetetraacetic acid disodium salt (EDTA), phenyl isothiocyanate (PITC), and dithiothreitol (DTT) were obtained from Wako Pure Chemical Industries (Osaka, Japan). A strong cation-exchange resin (AG50×8) was obtained from Bio-Rad Laboratories (Hercules, CA).

Preparation of Gluten Hydrolysate on an Industrial Scale. A wheat gluten hydrolysate was prepared on an industrial scale as described previously (*7*, *8*). This preparation contains 0.4 mmol/g of the pyroglutamyl peptide (*8*, *9*).

In Vitro Digestion of the Gluten Hydrolysate. The gluten hydrolysate was further digested with porcine pepsin and pancreatin in vitro to obtain the indigestible peptide. The gluten hydrolysate (0.5 g) was dissolved in 50 mL of 0.1 M HCl and digested with 5 mg of pepsin at 37 °C for 3 h in the presence of 15 mg of thimol. After the pepsin digestion, the reaction mixture was combined with 1 M NaOH to yield a pH 8.0 sample and then digested with 20 mg of pancreatin for 24 h. The digest was stored at −20 °C.

Isolation of Indigestible Pyroglutamyl Peptide. Indigestible pyroglutamyl peptide was isolated from the pepsin−pancreatin digest by the method described previously (*8*). The strong cation exchanger (AG50×8) was packed into a mini-spin column (15 × 5 mm, i.d., AB-1150, Atto, Tokyo, Japan) and washed with 50% ethanol and equilibrated with 50 mM formic acid. A 100 μL volume of the pepsin−pancreatin digest was applied to the mini-spin column and eluted with 100 μL of 50 mM formic acid (3 times). Elution was performed by centrifugation at 600 rpm for 1 min. The nonadsorbed fraction was dried under vacuum and redissolved in 50 μL of distilled water.

The pooled indigestible pyroglutamyl peptide fraction (200 μL) was subjected to size-exclusion chromatography (SEC) by using a PolyHYDROXYETHYL A column (200 × 9.4 mm i.d., 5 μm, 200 Å pore size, PolyLC, Columbia, MD) equilibrated with 50 mM formic acid at 2 mL/min (*11*, *12*). Before injection to the column, the sample was clarified by centrifugation at 12 000 rpm for 3 min and filtration with a Column Guard (0.45 μm, Millipore, Bedford, MA). Absorbance at 216 nm was monitored. The SEC fractions were further fractionated by reversed-phase (RP) HPLC by using a Cosmosil type MS C18 (250 × 4.6 mm, i.d., Nacalai tesque). Up to 2 mL of the sample was injected the column equilibrated with 0.1% TFA solution. After elution of the nonadsorbed materials, the adsorbed materials were eluted with a liner gradient of acetonitrile from 0 to 40% over 15 min in the presence of 0.1% TFA at 1 mL/min. Absorbance at 214 nm was monitored. The column was maintained at 40 °C.

Pyroglutamate Aminopeptidase Digestion. The pyroglutamyl peptides were digested with 0.2 mU of the porcine or bacterial pyroglutamate aminopeptidase at 37 °C for 3 h in 50 μL of 10 mM Tris-HCl buffer, pH 7.4 containing 1 mM EDTA, 5 mM DTT, and 50% glycerol or 50 μL of sodium phosphate buffer, pH 7.2, containing 10 mM DTT and 1 mM EDTA, respectively. The reaction was terminated by adding 50 μL of 10 mM HCl and then immediately dried under vacuum.

Isolation of Phenylthiocarbamyl Peptides. The pyroglutamyl aminopeptidase digest was reacted with phenyl isothiocyanate (PITC) as described previously (*13*). The resultant phenylthiocarbamyl (PTC) peptides were purified by RP-HPLC by using a Superspher 100RP-18(e) (250 × 4 mm i.d., Merck, Darmstadt, Germany) column equilibrated with 150 mM ammonium acetate buffer, pH 6.0, containing 5% acetonitrile at 0.8 mL/min. Elution was performed by a binary linear gradient with the equilibrium buffer (A) and 60% (v/v) acetonitrile (B). The gradient profiles were as follows: 0−2 min, 0% B; 2−20 min, 10−47.5% B; 20−25 min, 47.5−100% B; 25−37.5 min, 100% B; 37.5−50 min, 0% B. The column was maintained at 40 °C. Absorbance at 254 nm was monitored.

Amino Acid Analysis. Vapor phase HCl hydrolysis was performed by the method of Bidlingmeyer et al. (*14*). The amino acids were derivatized with PITC and the PTC amino acids were resolved by the same condition for the PTC peptide as described above.

Sequence Analyses of Peptide. Aliquot of the PTC peptide fraction was dried under vacuum to remove ammonia. The residue was added with 20 μL of a redrying solution consisting of methanol, water, and TEA at 7:1:2 and redried under vacuum. Then the PTC peptide was dissolved 50 μL of 37% acetonitrile and applied to an automatic peptide sequencer based on the Edman degradation method (PPSQ-10 or PPSQ-21; Shimadzu, Kyoto, Japan). Programs of the peptide sequencers were changed to start from the cleavage reaction with TFA as described in the previous paper (*15*).

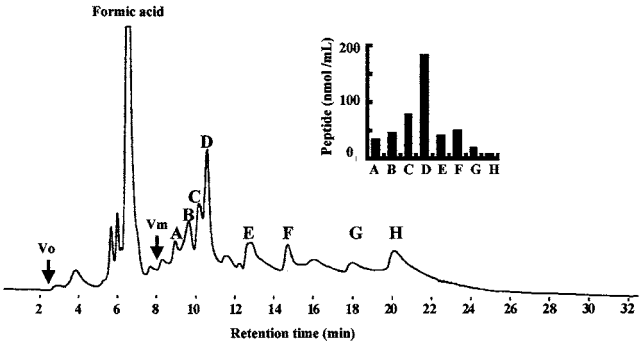

Figure 1. Fractionation of pyroglutamyl peptides in SEC mode. Peptide peaks marked with A–H were collected. Inset: Peptide contents are expressed as the content of constituting amino acids. Key: V_o, void volume, V_m, exclusion limit.

The sequences of some pyroglutamyl peptides were directly determined by fast-atom bombardment ionization tandem mass spectrometry (FAB-MS/MS) in positive ion mode by using a JEOL JMS-HX/HX110A four-sector tandem mass spectrometer equipped with an array detector. FAB was carried out using xenon as the primary beam with 6 keV energy, and the ion acceleration voltage was 10 kV. In the MS/MS experiment, high-energy collision-induced dissociation was performed by introducing helium to the collision cell, floated at 8 kV, until the intensity of the precursor ion decreased to 1/3 of the initial value. Glycerol and a mixture of glycerol, thioglycerol, and small amount of TFA and 3-nitrobenzyl alcohol were used for the matrix. Xe was used as primary particle. The sequences of the peptides were verified by analysis of product ion spectrum of the molecular-related ion. The product ions of peptides are named according to the proposal by Roepstorff and Fohlman (*16*).

RESULTS

Purification of Pyroglutamyl Peptides. As shown in **Figure 1**, the indigestible pyroglutamyl peptides were eluted after the exclusion limit of the column in the SEC mode (V_m), whereas the peaks between the column void and exclusion limit ($V_o - V_m$; approximately 3−8 min) contained only negligible amounts of peptide. These facts indicate that the pyroglutamyl peptides interacted with the stationary phase of the column. The SEC fractions (A−H) were further subfractionated by RP-HPLC (**Figure 2**). For the fr. D (fr. = fraction), a large peptide peak (fr. D-1) was eluted with 0.1% TFA after the elution of formic acid. For the other fractions, peptide peakes (marked with a number) were eluted by increasing the portion of acetonitrile. As shown in **Figure 3**, fr. D-1 yielded only glutamic acid by HCl hydrolysis, which indicates that fr. D-1 consists of pyroglutamic acid, glutamine, and its derivatives. The amino acid composition of the peptide peaks is shown in **Table 1**. The peptide fractions except for fr. D-1 consisted of proline and the other amino acids.

Sequence Analyses of Peptides. Pyroglutamyl peptides in fr. D-1 and C-1 were digested with the porcine pyroglutamate aminopeptidase. As shown in **Figure 4**, only glutamine was liberated from the fr. D-1 by the pyroglutamate aminopeptidase digestion, which suggests the presence of pyroGlu-Gln. The FAB-MS analysis also supports the presence of pyroGlu-Gln, while free pyroGlu and pyroGlu-$(Gln)_{n>2}$ were not detected (**Figure 5**). The other positive ion peaks marked with an asterisk

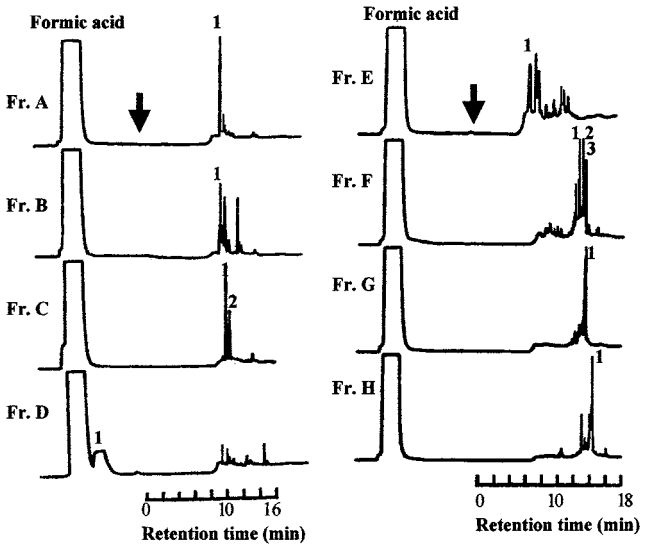

Figure 2. Subfractionation of pyroglutamyl peptides by RP-HPLC by using the 0.1% TFA–acetonitrile system. Fr. A–H: SEC fractions as shown in **Figure 1**. Peptide peaks marked with a number were collected. Arrows indicate the start of the gradient elution program.

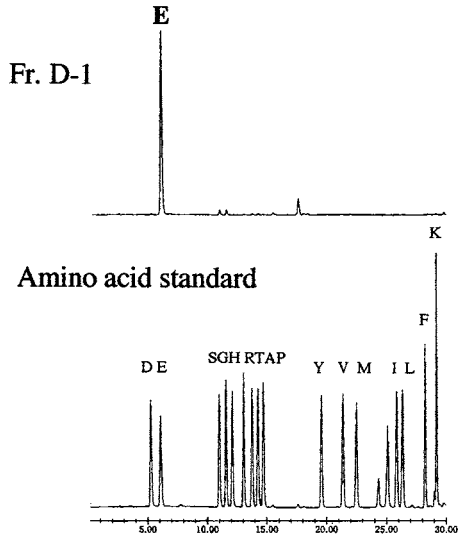

Figure 3. Amino acid analysis of fr. D-1. Only glutamic acid (E) was liberated by the HCl hydrolysis.

Table 1. Amino Acid Composition of Pyroglutamyl Fractions Prepared by Reversed Phase HPLC As Shown in **Figure 2** (%)[a]

	A-1	B-1	B-2	C-1	C-2	D-1	E1	F-1	F-2	G-1	H-1
Asp	16										
Glu	57	80	48	75	57	100	64	68	45	43	30
Ser											19
Gly	8				29				20	11	6
Ala			26								
Pro	19	20	26	25	14		36	21	25	25	31
Ile								11	10		
Phe										21	14

[a] The absence of a value indicates that a negligible amount was detected.

could not be ascribed to the peptide consisting of pyroglutamic acid, glutamine, and glutamic acid. Together with the compositional data, it can be concluded that pyroGlu-Gln is the major peptide constituent of fr. D-1. For fr. C-1, the peak marked by an arrow (**Figure 4**) yielded a sequence of Gln-Pro-Gln, whereas the other peaks did not yield any significant sequence. The FAB-

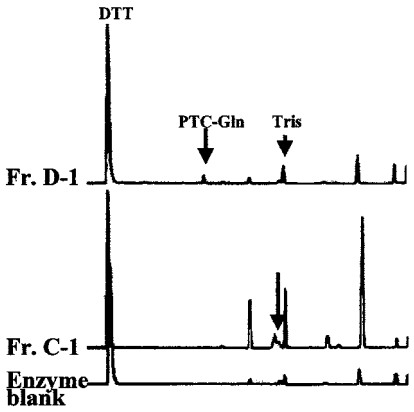

Figure 4. Isolation of the PTC amino acid/peptide in the porcine pyroglutamyl aminopeptidase digests of fr. D-1 and C-1. The peak marked with an arrow represents PTC peptide (C-1). DTT and Tris were derived from the enzyme reaction buffer.

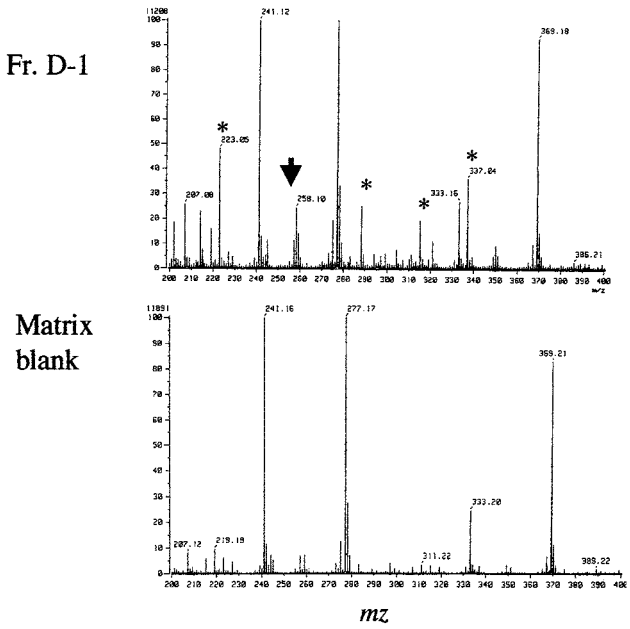

Figure 5. FAB-MS analysis of fr. D-1. The positive ion peak corresponding to pyroGlu-Gln (*m/z* 258) is marked with an arrow. Other positive ion peaks are marked with asterisks.

MS/MS analysis of the undigested fr. C-1 yielded a major positive ion peak at *m/z* 483 corresponding to pyroGlu-Gln-Pro-Gln (**Figure 6a**), which yielded product ions corresponding to pyroGlu-Gln, pyroGlu-Gln-Pro, and Gln-Pro and low mass immonium ions of Gln, pyroGlu, and Pro (**Figure 6b**). Together with these facts, it can be concluded that pyroGlu-Gln-Pro-Gln is a major constituent of fr. C-1.

Other pyroglutamyl peptide fractions were digested with the pyroglutamyl aminopeptidase from *Pyrococcus furiosus,* because the supplier changed source for pyroglutamyl aminopeptidase from porcine kidney. As for fr. F-3, three peaks marked with the arrows yielded sequences of Gln-Pro-Leu, Gln-Phe-Pro-Gln, and Ser-Phe-Pro-Gln, respectively (**Figure 7**). For the other fractions, only one peak marked with the arrow yielded one sequence. The identified sequences are summarized in **Table 2**.

The content of pyroglutamyl peptides is roughly estimated on the basis of glutamic acid content in the HCl hydrolysate. As shown in **Table 2**, pyroGlu-Gln was a major constituent and the other peptides accounted for a few percent.

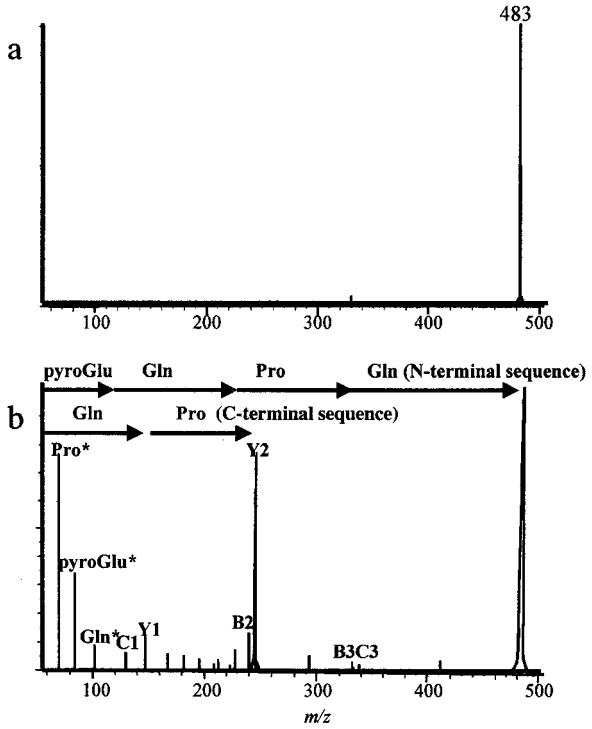

Figure 6. FAB-MS/MS analyses of fr. C-1. The major peak at *m*/*z* 483 (a) was applied to MS/MS analysis (b). Asterisks indicate low molecular immonium ions.

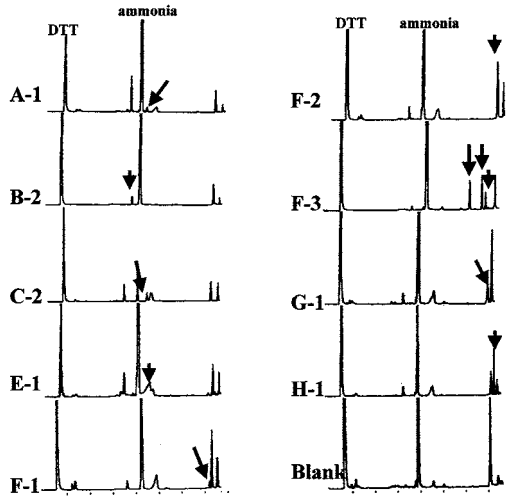

Figure 7. Isolation of PTC peptides in the bacterial pyroglutamyl aminopeptidase digests. Peaks marked with arrows represent PTC peptides. The sequences of these peptides are summarized in **Table 2**.

DISCUSSION

As shown in **Table 2**, the indigestible peptides in the wheat gluten hydrolysate were similar in structure and molecular size. It was difficult to separate these closely related small peptides only by the RP-HPLC by using the 0.1% TFA−acetonitrile system, a common method for peptide isolation. To solve this problem, we used a PolyHYDROXYETHL A column for the first fractionation of the pyroglutamyl peptides. This resin is a silica-based material coated with hydrophilic polymer, poly(2-hydroxyethylaspartamide), which has a weak cationic character (*11*, *12*). It was first introduced for hydrophilic interaction chromatography (*17*). In addition, peptide of molecular weight less than 1200 can be also fractionated by the same column on the basis of peptide size, when 50 mM formic acid is used for the mobile phase (*11*, *12*). The present study demonstrates that the pyroglutamyl peptides in the wheat gluten hydrolysate were eluted in eight fractions (A−H) after the exclusion limit of the column possibly due to the weak anion exchange effect (**Figure 1**). We also found the pyroglutamyl peptides in the other protein hydrolysates were eluted after the exclusion limit, while peptides with open N-terminal amino group were eluted on the basis of their peptide size. Therefore, the PolyHYDROXYETHL A column chromatography in the SEC mode is useful for the isolation and fractionation of the short chain pyroglutamyl peptides.

Table 2. Summary of Structure and Recovery of Pyroglutamyl Peptides in a Wheat Gluten Hydrolysate

fraction	sequence	origin[a]	%
A-1	pyroGlu-Asn-Pro-Gln	a	2
B-1	pyroGlu-Gln-Gln-Pro-Gln	e	1
C-1	pyroGlu-Gln-Pro-Gln	b, c, e	3
C-2	pyroGlu-Gln-Pro-Gly-Gln-Gly-Gln	d	5
D-1	pyroGlu-Gln	a–e	76
E-1	pyroGlu-Gln-Pro	a–e	5
F-1	pyroGlu-Ile-Pro-Gln	c	>1
F-2	pyroGlu-Ile-Pro	c	>1
F3-1	pyroGlu-Gln-Pro-Leu	b	>1
F3-2	pyroGlu-Gln-Phe-Pro-Gln	c	>1
F3-3	pyroGlu-Ser-Phe-Pro-Gln	b	>1
G-1	pyroGlu-Phe-Pro-Gln	c	3
H-1	pyroGlu-Gln-Pro-Pro-Phe-Ser	e	3

[a] Key: a, α/β-gliadin (*18*); b; γ-gliadin (*19*); c, γ-gliadin B (*20*); d, glutenin high molecular weight subunit (*21*); e, glutenin low molecular subunit (*22*, *23*).

For the sequence analysis based on Edman degradation, the N-terminal pyroglutamyl residue must be removed. However, pyroglutamate aminopeptidase digestion of the peptide having pyroGlu-Gln- motif would yield the peptide with N-terminal glutamine, which would be reconverted to pyroglutamyl peptide due to cyclization of newly appeared N-terminal glutamine residue (*9*). In addition, some peptides in the pyroglutamate aminopeptidase digests were eluted in the nonadsorbed fraction with buffer components by the RP-HPLC by using the 0.1% TFA−acetonitrile system. To solve these problems, we used a modified method of a general amino acid analysis based on derivatization with PITC; first, peptides in the digests are reacted with PITC. Second, the PTC peptide is purified by RP-HPLC by using the ammonium acetate buffer−acetonitrile system and detected specifically at 254 nm. Third, the isolated PTC peptides are analyzed by the automatic peptide sequencer with a slightly modified program that omitted the first step of PITC derivatization from the general method. The validity of this sequencing approach was confirmed by the FAB-MS/MS analysis (**Figures 5** and **6**). It is worthy to note three PTC peptides with different sequences were isolated from fr. F-3 (**Figure 7**), which could not be separated by the common RP-HPLC (**Figure 2**).

The indigestible pyroglutamyl peptides identified in this study can be derived from wheat storage proteins (*18*−*23*). pyroGlu-Gln-Pro-Pro-Phe-Ser is a part of the Ig-E binding epitope (*24*), and pyroGlu-Gln-Pro-Gly-Gln-Gly-Gln is a repeated motif in the glutenin high molecular weight subunit (*21*). If these indigestible peptides are adsorbed without further digestion, they might bind the IgE on mast cells and consequently suppress antigen-induced cross-linking of the IgE receptor. pyroGlu-Gln is the most abundant peptide in the indigestible pyroglutamyl peptide fraction. The poly-glutamine motif up to octomers in size is distributed in the wheat storage proteins (*18*−*23*). However, the pyroglutamyl peptide derived from the poly-glutamine motif longer than a dimer could not be detected in

this study. Therefore, it can be assumed that the proteases used in this study can cleave the peptide bond between glutamine residues in the pyroglutamyl peptides but cannot cleave the peptide bond between pyroglutamic acid and glutamine residues. On the other hand, the indigestible pyroglutamyl peptides longer than pyroGln-Gln contained proline and/or glycine residues (**Table 2**). In a previous study, we observed that the recoveries of proline and glycine from protein by exhaustive proteolysis were significantly lower than expected values, whereas sufficient recoveries were obtained for other amino acids (*13*). Then the presence of proline and glycine may also contribute to the indigestibility of the pyroglutamyl peptides. This information would be useful to design indigestible peptides.

In the previous study, we reported that pyroglutamyl peptides are widely distributed in enzymatic hydrolysates prepared on an industrial scale (*9*). These preparations are now being used for beverage, infant, and clinical formulas. These products have been successfully used to improve the nutritional state (*25*, *26*), moderate allergic reactions (*27*, *28*), and so on. Then, it could be assumed that the pyroglutamyl peptides in these preparations might not have serious side effects on humans by short-term oral administration under normal conditions. On the other hand, it has been demonstrated that some short-chain pyroglutamyl peptides in animal tissues have significant biological activities (*29*−*35*). Recently, some pyroglutamyl peptides (pyroGlu-Pro-Ser, pyroGlu-Pro, pyroGlu-Pro-Glu, and pyroGlu-Pro-Gln) were suggested to have a glutamate-like taste (*10*). Therefore, there is a possibility that the pyroglutamyl peptides unexpectedly induced during food processing might have some biological effects (including beneficial effects) on humans by oral administration under certain conditions. In addition, oral administration of pyroglutamic acid has been demonstrated to improve the age-associated memory impairment by a double-blind placebo-controlled randomized study (*36*), which suggests that oral administration of pyroglutamic acid might have some biological effect on the human nervous system. Therefore, we should also pay attention to pyroglutamic acid, which might be potentially liberated from the pyroglutamyl peptide.

The structural information and analytical procedures developed in this study might be important to access the health effects of the indigestible pyroglutamyl peptide. Now, further studies in vivo on the digestibility and the absorption of the pyroglutamyl peptide in food-graded peptide preparation are in progress.

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