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Occurrence of the Free and Peptide Forms of Pyroglutamic Acid in Plasma from the Portal Blood of Rats That Had Ingested a Wheat Gluten Hydrolysate Containing Pyroglutamyl Peptides

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In order to determine pyroglutamic acid levels in plasma, we developed a method based on precolumn derivatization of the carboxyl group of pyroglutamic acid with 2-nitrophenylhydrazine. Eight-week-old male SD strain rats were administered 200 mg of an acidic peptide fraction obtained from a commercial wheat gluten hydrolysate containing 0.63 mmol/g pyroglutamyl peptide. After administration, significant amounts of free pyroglutamic acid were observed in the ethanol-soluble fraction of the plasma from the portal vein. In addition, pyroglutamate aminopeptidase digestion of the ethanol-soluble fraction liberated significant amounts of pyroglutamic acid, which indicated the presence of the pyroglutamyl peptide. The presence of the pyroglutamyl peptide in the plasma was further confirmed by size exclusion chromatography. The levels of free and peptide forms of pyroglutamic acid increased significantly and reached a maximum (approximately 40 nmol/mL) at 15 and 30 min after administration, respectively.

KEYWORDS: Pyroglutamic acid; wheat gluten hydrolysate; pyroglutamyl peptides

INTRODUCTION

Compared with proteins, peptides present in enzymatic hydrolysates of food proteins show higher solubility and adsorption rates and lower antigenicity. In addition, the enzymatic hydrolysate of proteins can be considered as a reliable source of unstable amino acids such as glutamine, cysteine, and so on. Therefore, enzymatic hydrolysates of food proteins have been used in sports drinks, infant and enteral formulas, and so on in order to improve nutritional values and decrease antigenicity (*1*−*4*). Furthermore, enzymatic hydrolysates have been used as a source of peptides that have health-promoting activities such as the enhancement of intestinal adsorption of calcium, growth of lactic acid bacteria in the gut, and moderation of hypertension and hypercholesterolemia (*5*−*13*). Recently, a variety of enzymatic hydrolysates of food proteins have been commercially available.

It is well-known that L-2-pyrrolidone-5-carboxylic acid (pyroglutamic acid) is readily formed by heat treatment of glutamine solution. The amino-terminal glutaminyl residue of the peptide is also converted to a pyroglutamyl residue, which induces formation of the pyroglutamyl peptide. The presence of pyroglutamyl peptides in enzymatic hydrolysates of wheat gluten, cow milk casein, whey protein, and so on has been demonstrated (*15*−*17*). We have also demonstrated that some pyroglutamyl peptides present in a gluten hydrolysate resisted exhaustive in vitro peptidase digestion (*15*, *17*). These facts suggest that some pyroglutamyl peptides might resist in vivo digestion and may be absorbed into the blood circulation system in the form of peptides. However, there is no data on the absorption and metabolic fate of the pyroglutamyl peptide present in the enzymatic hydrolysate of food proteins.

Gas−liquid chromatography has been used to determine relatively high levels (mM quantities) of plasma pyroglutamic acid present in animal (*18*−*20*). However, tedious clarification steps based on strong cation-exchange chromatography and liquid−liquid partitioning are required for removing interfering substances prior to the methylation and acetylation steps in order to increase the volatility of pyroglutamic acid. Therefore, there is a demand for a simple and sensitive method for the determination of plasma pyroglutamic acid.

The objective of this study was to examine the presence of the free and peptide forms of pyroglutamic acid in the portal blood of animals after the ingestion of an enzymatic hydrolysate of food proteins containing pyroglutamyl peptides. For determining pyroglutamic acid present in rat plasma, we developed a method based on precolumn derivatization of the carboxyl group of pyroglutamic acid with 2-nitrophenylhydrazine.

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MATERIALS AND METHODS

Reagents. *Pyrococcus furiosus* pyroglutamate aminopeptidase was obtained from Takara Bio (Ohtsu, Japan). Trifluoroacetic acid (TFA), dithiothreitol (DTT), disodium ethylendiaminetetraacetate (EDTA), and acetonitrile (HPLC grade) were obtained from Wako Pure Chemicals (Osaka, Japan). Other reagents that were used were of analytical grade or of a more superior grade.

Preparation of Gluten Hydrolysate. A commercially available food-grade enzymatic hydrolysate of wheat gluten was used. Peptides in this preparation (100 g) were further fractionated on the basis of the amphoteric nature of sample peptides by using a previously described method (*21*). An acidic peptide fraction with p*I* less than 3.1 was collected.

Animal Experiment. This study was performed in accordance with the guidelines for animal experimentation of Kyoto Prefectural University. Four 8-week-old male SD strain rats were purchased from SLC Japan (Shizuoka, Japan). A midline abdominal incision was made for about half the length of the abdomen posterior to the xiphoid cartilage under pentobarbital sodium (40 mg/kg, ip; Dainippon Pharmaceutical, Osaka, Japan) anesthesia. A heparinized silicone tube (0.5 mm i.d., 1.0 mm o.d.) was inserted into the portal vein and fixed with an adhesive (Aronalpha; Toagosei, Tokyo, Japan). The rats were fed on a commercial diet for 1 week after the surgery. After fasting for 16 h, 2 mL of the 10% (w/v) acidic peptide fraction was intragastrically administered to the rats (average body weight, 274.2 g) using a 15G curved blunt-ended needle cannula. Approximately 300 μL of blood was collected from the portal vein through the heparinized silicone tube before and at 15, 30, 60, and 120 min after administration. Plasma was prepared and immediately mixed with 3 volumes of ethanol. The resulting proteinous precipitate was removed by centrifugation at 12 000 rpm for 5 min. The ethanol-soluble fraction, which may contain peptides with molecular weight less than 5000, was collected and stored at −80 °C until use.

Size Exclusion Chromatography (SEC). An aliquot of the ethanol-soluble fraction that was obtained at 30 min after administration was fractionated by SEC using a Superdex peptide HR 10/30 column (Amersham Biosciences, Piscataway, NJ). The column was equilibrated with 0.1% TFA containing 30% acetonitrile at a flow rate of 0.5 mL/min. Aliquots (400 μL) of the ethanol-soluble fraction prepared from the rat at 30 min after administration of the acidic peptide fraction were dried under vacuum, dissolved in 200 μL of 0.1% TFA containing 30% acetonitrile, and injected into the column. Fractions were collected every 1 min.

Pyroglutamate Aminopeptidase Digestion. Gluten hydrolysate and its acidic peptide fraction were dissolved in distilled water to give a 0.05% (w/v) solution. Fifty microliters of the solution was pipetted into 1.5 mL centrifuge tubes and dried under vacuum. Aliquots (120 μL) from the ethanol-soluble fraction of the plasma and the SEC fractions were also dried in the tubes. These samples were dissolved in 120 μL of 100 μM sodium EDTA, pH 7.0, containing 5 mM DTT. Aliquots (50 μL) of the sample solutions were used for determining the amount of free pyroglutamic acid. Another 50 μL of the sample solution was digested with 0.3 mU of pyroglutamate aminopeptidase at 50 °C for 60 min for determining the amount of the pyroglutamyl peptide. The reaction was terminated by cooling the mixture with ice.

Solid-Phase Extraction by a Strong Cation-Exchange Resin. To remove amino acids and peptides from the free and peptide forms of pyroglutamic acid, we carried out solid-phase extraction using a spin column (5 mm × 5 mm i.d.; AB-1150, Atto, Tokyo, Japan) packed with a strong-cation exchange resin (AG50W×8, Bio-Rad Laboratories, Hercules, CA) as described previously (*14*, *17*) but with slight modifications. The resin was packed into the spin column in 50% ethanol. The column was placed into a 1.5 mL centrifugation tube. Elution was carried out by centrifugation at 12 000 rpm for 1 min. The resin was equilibrated with 100 μL of 10 mM HCl (twice) after washing with 50% ethanol (twice). The pyroglutamate aminopeptidase digest or nondigest was then applied on the column. The free and peptide forms of pyroglutamic acid were eluted from the column. The column was washed with 200 μL of 10 mM HCl (thrice). These effluents were combined and dried under vacuum.

Table 1. Amino Acid Composition of Gluten Hydrolysate and Its Acidic Peptide Fraction (mol %)

	starting material	acidic peptide fraction		starting material	acidic peptide fraction
Asp	2.6	2.8	Pro	15.5	16.7
Glu	38.8	43.7	Tyr	1.9	1.5
Ser	6.7	6.1	Val	3.7	3.1
Gly	6.5	5.9	Met	1.1	0.9
His	1.5	0.7	Ile	2.8	2.3
Arg	2.0	1.4	Leu	6.1	5.3
Thr	2.6	2.3	Phe	4.0	4.4
Ala	3.2	2.5	Lys	1.1	0.3

Ion Chromatography. The AG50W×8-unbound fraction was redissolved in 200 μL of distilled water and used as the sample solution for ion chromatography. In the ion-exclusion mode, the sample was injected into an HPICE-AS1 column (Dionex, Sunnyvale, CA) that had been equilibrated with 1 mM octanesulfonic acid at a flow rate of 0.5 mL/min (*14*). The column effluent was passed through a chemical suppressor (AMS-ICE, Dionex) regenerated with 10 mM tetrabutylammonium hydroxide to suppress the conductivity of the eluent. The suppressed conductivity was monitored. In the anion-exchange mode, the same sample was injected into an Ion Pac AS4A-SC column (Dionex) equilibrated with 1.4 mM $Na_2B_4O_7$ at a flow rate of 1 mL/min. The column effluent was passed through another type of chemical suppressor (MMAM-II, Dionex) regenerated with 100 mM H_2SO_4. The suppressed conductivity and absorbance at 214 nm were monitored.

Derivatization of Pyroglutamic Acid. The carboxyl group of pyroglutamic acid was derivatized by the method of Miwa et al. (*22*, *23*) using a fatty acid label kit (YMC, Kyoto, Japan). The AG50W×8-unbound fraction was redissolved in 60 μL of 50% ethanol and mixed with 40 μL of 0.02 M 2-nitrophenylhydrazine hydrochloride (reagent A in the kit) and 40 μL of 0.25 M 1-ethyl-3-(3-dimethylaminopropyl-9-carbodiimide hydrochloride) (reagent B) and then reacted at 60 °C for 20 min. Subsequently, 1 M KOH (reagent C) was added, and the reaction continued at 60 °C for 15 min. The reaction was terminated by the addition of 180 μL of 50 mM phosphoric acid; this resulted in a pH value less than 6. The resultant 2-nitrophenylhydrazide was resolved by reversed phase HPLC using a Cosmosil MS II 5C18 column (250 × 4.6 mm, i.d., Nacalai Tesqu) and by binary solvent gradient elution. The solvents consisted of 50 mM sodium phosphate buffer, pH 6.0, containing 10% acetonitrile (solvent A) and 60% acetonitrile (solvent B). The column was equilibrated with 15% B. The elution program was as follows: 0−15 min, B 15%; 15−25 min, B 100%; 25−35 min, B 15%. During elution, the flow rate was 1 mL/min. The column was maintained at 45 °C. The absorbance at 400 nm was monitored.

Amino Acid Analysis. Amino acid analysis was performed by the method of Bidlingmeyer et al. (*24*) with a slight modification (*17*).

Statistical Analysis. The increase in the free and peptide forms of pyroglutamic acid present in the plasma after the ingestion of the acidic peptide fraction of a gluten hydrolysate was analyzed by one-way ANOVA using StatView 4.11 (Abacus Concepts Inc., Berkeley, CA). Significant differences between the samples were evaluated by Fisher's PLSD test.

RESULTS

Composition of the Gluten Hydrolysate. An acidic peptide fraction with p*I* less than 3.1 was prepared from the commercial gluten hydrolysate by ampholyte-free preparative isoelectric focusing. Recovery of the acidic peptide fraction was 45.3% on a weight basis. As shown in **Table 1**, the composition of the acidic peptide fraction is similar to that of the starting material except for the higher glutamic acid content. The contents of the free and peptide forms of pyroglutamic acid in the wheat gluten hydrolysate and its acidic peptide fraction were evaluated by anion-exchange ion chromatography. As shown in **Figure 1**, significant amounts of pyroglutamic acid were

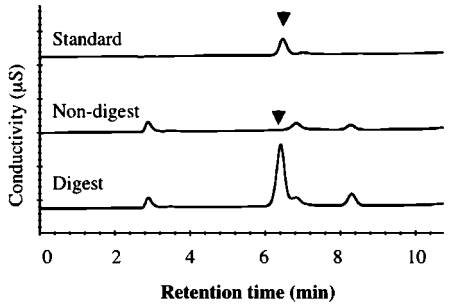

Figure 1. Detection of the free and peptide forms of pyroglutamic acid in the acidic peptide fraction by anion-exchange chromatography. AG50W×8-unbound fraction was dissolved into 200 μL of distilled water, and 20 μL was injected. See Materials and Methods section for experimental details. Arrow indicates the elution position of pyroglutamic acid.

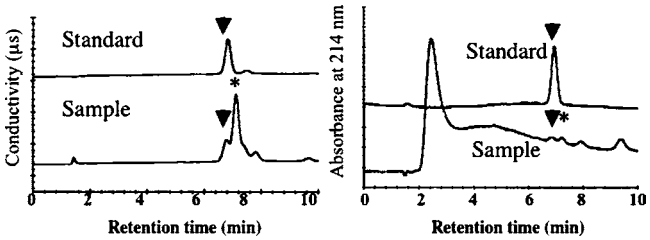

Figure 2. Chromatograms of pyroglutamate aminopeptidase digest of the ethanol-soluble fraction at 30 min after administration. Elution was monitored by suppressed conductivity (**A**) and absorbance at 214 nm (**B**). Arrow indicates the elution position of pyroglutamic acid. Asterisk indicates the lactic acid peak.

liberated by pyroglutamate aminopeptidase digestion of the acidic peptide fraction, while only negligible amounts of pyroglutamic acid were observed in the nondigest. Thus, this preparation predominantly contained the peptide form of pyroglutamic acid. The pyroglutamyl peptide contents in the starting material and the acidic peptide fraction were 0.21 and 0.63 mmol/g, respectively. On the other hand, only negligible amounts of pyroglutamyl peptide were contained in the basic peptide fraction.

Methods for Determining the Level of Pyroglutamic Acid in the Plasma. Figure 2 shows representative chromatograms from anion-exchange chromatography of the ethanol-soluble fraction of plasma from the rat at 30 min after administration. When conductivity measurements were used to detect the presence of pyroglutamic acid, there was interference by a large peak of lactic acid (marked with an asterisk) that followed the pyroglutamic acid peak. Using UV detection, the lactic acid peak became smaller: however, extensive baseline drift occurred. In the case of the ion-exclusion mode, the results were more disappointing (data not shown). Then, an alternative approach based on the precolumn derivatization technique was used. As shown in **Figure 3**, it was possible to obtain baseline resolution of the 2-nitrophenylhydrazide of pyroglutamic acid from the reagents and other organic acids present in plasma. When 15 nmol of pyroglutamic acid was added to 100 μL of the ethanol-soluble fraction, the pyroglutamic acid recovery was 102%. Good linearity ($r^2 = 1.00$) was observed between the peak area and the pyroglutamic acid content in the concentration range from 0 to 1 nmol.

Presence of the Pyroglutamyl Peptide in Plasma. As shown in **Figure 3**, pyroglutamate aminopeptidase digestion of the ethanol-soluble fraction from the rat at 30 min after administration increased pyroglutamic acid content from 29.7 nmol/mL of plasma to 70.8 nmol/mL: this suggested the presence of the

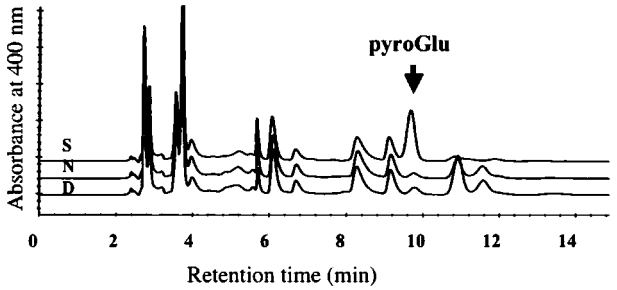

Figure 3. Chromatograms of 2-nitrophenylhydrazides of authentic pyroglutamic acid (S), the ethanol-soluble fraction (N), and its pyroglutamate aminopeptidase digest (D). Arrow indicates the elution position of pyroglutamic acid. Twenty microliters of the reaction mixture was injected.

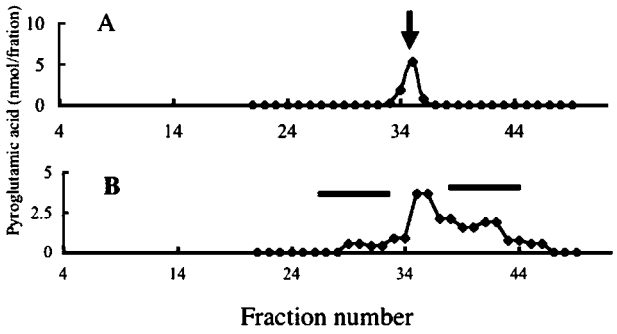

Figure 4. Liberation of pyroglutamic acid from aliquots of SEC fractions. Authentic pyroglutamic acid (**A**) and the ethanol-soluble fraction at 30 min after administration (**B**) were subjected to SEC. Aliquots of SEC fractions were digested by pyroglutamyl aminopeptidase and analyzed for their pyroglutamic acid content. Arrow indicates the elution position of pyroglutamic acid.

pyroglutamyl peptide in the plasma. The ethanol-soluble fraction was subjected to SEC. As shown in **Figure 4**, when some of the fractions that eluted before and after the free pyroglutamic acid (arrow) were subjected to pyroglutamate aminopeptidase digestion, they yielded pyroglutamic acid (bars in **Figure 4B**): this confirms the presence of the pyroglutamyl peptide in the plasma.

Determination of Free and Peptide Forms of Pyroglutamic Acid in Plasma. The content of the pyroglutamyl peptide was determined by subtracting the amount of free pyroglutamic acid from that of total pyroglutamic acid present in the pyroglutamate aminopeptidase digest. As shown in **Figure 5**, the free and peptide forms of pyroglutamic acid present in the plasma significantly increased and attained maximum levels at 15 and 30 min after the administration, respectively ($P < 0.05$).

DISCUSSION

Pyroglutamic acid liberated from enzymatic hydrolysates of proteins has been determined by ion-exclusion chromatography in conjunction with UV or suppressed conductivity for detection (*14*, *15*). This technique requires prefractionation with reversed phase chromatography or additional ion-exclusion chromatography. On the other hand, the present method based on anion-exchange chromatography with suppressed conductivity detection allows direct injection of the wheat gluten hydrolysate for determining the content of pyroglutamic acid. However, due to the presence of large amounts of interfering substances, these methods could not be applied for determining the μM levels of pyroglutamic acid present in the plasma of animals that had ingested the gluten hydrolysate and even its acidic peptide

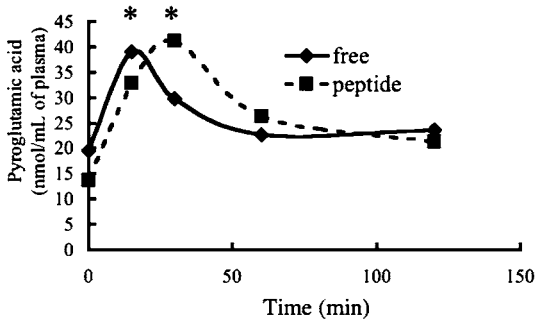

Figure 5. Plasma levels of free and peptide forms of pyroglutamic acid after administration of the acidic peptide fraction containing 126 μmol of pyroglutamyl peptide. Asterisks indicate a significant increase from the initial value $P < 0.05$ ($n = 4$).

fraction containing approximately three times higher pyroglutamyl peptide. Since pyroglutamic acid is weakly retained on the anion-exchange column even when a low ionic strength solution is used as the eluent, it was difficult to improve the resolution by optimizing the elution conditions.

Thus, we chose an alternative approach based on precolumn derivatization of the carboxyl group of pyroglutamic acid with 2-nitrophenylhydrazine: this approach had been developed for the identification and determination of hydrophobic fatty acids by HPLC. The 2-nitrophenylhydrazides of these fatty acids can be easily separated from the derivatization reagents by using reversed phase chromatography by using eluents without pH control (*22*, *23*). However, in order to separate the 2-nitrophenylhydrazides of pyroglutamic acid from the reagents, pH control was critical. In addition, animal and human plasma contain considerable amounts of amino acids and peptides with carboxyl groups that can react with 2-nitrophenylhydrazine. Therefore, these components should be removed prior to the derivatization. For this purpose, we used the mini-spin column that was packed with a strong cation exchanger (AG50W$\times$8) and placed in a 1.5 mL centrifugation tube. To stabilize pyroglutamate aminopeptidase, the enzyme supplier recommends adding EDTA to the reaction mixture, which can liberate theoretical amounts of pyroglutamic acid from synthetic pyroglutamyl peptides (*15*). Our preliminary study using the anion-exchange chromatography revealed that pyroglutamate aminopeptidase can liberate constant amounts of pyroglutamic acid in the presence of EDTA concentrations greater than 100 μM. As shown in **Figure 3**, the presence of 100 μM EDTA did not interfere with the determination of pyroglutamic acid by reversed phase HPLC. On the basis of these findings, we have developed a method for determining the free and peptide forms of plasma pyroglutamic acid. All derivatization procedures, including solid-phase extraction, can be carried out in a 1.5 mL centrifugation tube. Therefore, multiple samples can be simultaneously and easily processed.

On the basis of reactivity to 2-nitrophenylhydrazine, retention time of its derivative, and susceptibility to pyroglutamate aminopeptidase, presence of significant amounts of free and peptide forms of pyroglutamic acid are demonstrated in the rat plasma before ingestion after fasting for 12 h, which might be derived from endogenous pyroglutamyl peptides and proteins. Furthermore, we demonstrate an increase in the free and peptide forms of pyroglutamic acid present in the plasma from the portal vein following administration of the acidic peptide fraction of the gluten hydrolysate. Subsequently, some pyroglutamyl peptides are degraded to free pyroglutamic acid in the digestive tract and/or blood. However, some pyroglutamyl peptides derived from the wheat gluten hydrolysate are absorbed into the circulatory system. This is the first report on the presence of a food-derived pyroglutamyl peptide in the blood. It has been demonstrated that considerable amounts of pyroglutamyl peptides are present in enzymatic hydrolysates of proteins prepared for commercial use (*14*−*17*). In some preparations, the content of the pyroglutamyl peptide is approximately 2 times higher than that in the acidic peptide fraction used in the present study. To the best of our knowledge, there is no report on the biological activity of food-derived pyroglutamyl peptides, while some endogenous pyroglutamyl peptides have significant biological activities (*25*−*33*). However, the potential biological activities of the food-derived pyroglutamyl peptide might have been overlooked in previous studies due to difficulty in detecting and identifying their primary structure by Edman degradation. Our method for specific and sensitive detection of the pyroglutamyl peptide present in the plasma would be a useful tool for resolving these problems.

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