Release of opioid peptides, gluten exorphins by the action of pancreatic elastase

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Abstract **The release of opioid peptides, gluten exorphins A, which have been isolated from the pepsin–thermolysin digest of wheat gluten, with gastrointestinal proteases was examined. High levels of gluten exorphin A5 (Gly–Tyr–Tyr–Pro–Thr) immunoreactive materials were detected in the pepsin–pancreatic elastase digest by a competitive ELISA. From this digest, gluten exorphin A5, B5 and B4 were isolated. This means that these peptides are released in the gastrointestinal tracts after ingestion of wheat gluten. The yield of gluten exorphin A5 in the pepsin–elastase digest was larger than that in the pepsin–thermolysin digest. The gluten exorphin A5 sequence is found 15 times in the primary structure of the high molecular weight glutenin. The region from which gluten exorphin A5 was released by the action of pancreatic elastase was identified using synthetic fragment peptides.**

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Key words: Opioid peptide; Gluten; Exorphin; Glutenin; Elastase

1. Introduction

We isolated four opioid peptides, Gly–Tyr–Tyr–Pro–Thr, Gly–Tyr–Tyr–Pro, Tyr–Gly–Gly–Trp–Leu and Tyr–Gly–Gly–Trp, from the pepsin–thermolysin digest of wheat gluten and named them gluten exorphin A5, A4, B5 and B4, respectively [1]. Among them, gluten exorphins A have peculiar structures and are rather selective for δ-receptors. Gluten exorphin A5 stimulated postprandial insulin release after oral administration in rats, and the effect was reversed by co-administration of naloxone [2]. However, it was not clear whether gluten exorphins A could be released in the gastrointestinal tracts.

Thus, to clarify whether gluten exorphin A5 is released with gastrointestinal proteases from wheat gluten, various digests were assayed for gluten exorphin A5 immunoreactive materials by a competitive ELISA. In this paper, we report the isolation of gluten exorphins A and B from the pepsin–pancreatic elastase digest of wheat gluten and the mechanism of release of gluten exorphins A. The sequence of this peptide is found 15 times in the primary structure of high molecular weight glutenin [3]. The 15 regions containing the gluten exorphin A5 sequence in wheat glutenin are classified into four groups according to the preceding residues. The region from which this peptide was released by the action of pancreatic elastase was identified using four synthetic tetradecapeptides.

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Abbreviations: MVD, mouse vas deferens; ODS, octadecyl silica; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; ELISA, enzyme-linked immunosorbent assay; KLH, keyhole limpet hemocyanin; PBS, phosphate-buffered saline

2. Materials and methods

2.1. Chemical and reagents

Wheat gluten was obtained from Goodman Fielder Mills, Ltd. Pepsin, trypsin, chymotrypsin and pancreatin were from Sigma Chemical Co. Porcine pancreatic elastase was from Elastin Products Co. Thermolysin was from Seikagaku Kougyo Co. Ltd. Naloxone was from USPC Inc. Other reagents used were reagent grade or better.

2.2. Preparation of gluten exorphin A5 antiserum

Coupling of gluten exorphin A5 to carrier protein was carried out by the following procedures. KLH (20 mg) and 7 mg of gluten exorphin A5 were dissolved in 2 ml of distilled water. This mixture (pH 7) was incubated with 40 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide at room temperature for 4 h. The solution was further incubated at 4°C overnight and then dialyzed against PBS (pH 7.4) at 4°C for 3 days. Gluten exorphin A5–KLH conjugate was emulsified in the same amount of Freund's complete adjuvant. A dose corresponding to 1 mg of gluten exorphin A5 was injected intradermally into each female rabbit. The immunization was repeated after 4 and 6 weeks at a dose corresponding to 0.5 mg of the peptide emulsified in an incomplete adjuvant. The serum was collected 1 week later.

2.3. Detection of gluten exorphin A5 immunoreactive materials by a competitive ELISA

To detect gluten exorphin A5 immunoreactive materials in the wheat gluten digests, the following competitive ELISA was performed. All procedures were done at room temperature. BSA–gluten exorphin A5 conjugates (1 mg/ml in 0.5 M $NaHCO_3$, pH 9.6, containing 0.2% NaN_3) were incubated in wells (100 μl/well) of microplates with 96 flat-bottomed wells (NUNC, 43954, Copenhagen, Denmark) for 2 h. The unfixed conjugates were removed by washing the plates with washing buffer (PBS containing 0.5% Tween 20 and 0.2% NaN_3). After this washing, blocking solution (1% BSA in PBS containing 0.2% NaN_3) was incubated in each well (100 μl/well) for 2 h to coat the surface of the well. After the above washing, 50 μl of a standard gluten exorphin A5 solution (0.1–100 mM peptide in washing buffer) or 50 μl of wheat gluten digests (0.1–10 mg/ml digest in washing buffer), and 50 μl of the gluten exorphin A5 antiserum (1:1000 dilution in washing buffer) was added to each well and their mixture was incubated for 2 h to form the gluten exorphin A5–antibody complex. After incubation, the wells were washed with washing buffer and anti-rabbit IgG-alkaline phosphatase (100 μl) was incubated in each well for 2 h to form the ternary complex of gluten exorphin A5–antibody linked with enzyme. After incubation, the wells were washed with washing buffer and then 100 μl of 2 g/l *p*-nitrophenyl phosphate disodium (Sigma) was added. After incubation for 20 min, the absorbance at 405 nm was measured with a microtiter reader (Nippon Bio-Rad, ELISA Reader Model 2550).

Preparation of BSA–gluten exorphin A5 conjugate was carried out by the following procedure. BSA (30 mg) and 10 mg of gluten exorphin A5 were dissolved in 4 ml of PBS. This mixture was incubated with 10 μl of 25% glutaraldehyde at room temperature for 2 h and then dialyzed against PBS (pH 7.4) at 4°C for 2 days.

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Table 1
The amount of gluten exorphin A5 immunoreactive materials in the digests of wheat gluten determined by a competitive ELISA

Digests	Gluten exorphin A5 immunoreactive materials (μmol/g)
Pepsin	N.D.[a]
Pepsin–trypsin	N.D.[a]
Pepsin–trypsin–chymotrypsin	N.D.[a]
Pepsin–pancreatin	8.4
Pepsin–pancreatic elastase	16.4
Pepsin–thermolysin	4.0

[a]Not detected.

2.4. Enzymatic digestion of wheat gluten and synthetic glutenin fragments

Wheat gluten (50 mg/ml solution) was digested with pepsin (0.5 mg/ml) in 0.02 N HCl (pH 2.0) for 5 h at 36°C. After digestion, the pH of the solution was adjusted with 1 N NaOH to 7.0. The solution was then boiled and centrifuged. The peptic digest was further digested with trypsin, chymotrypsin, pancreatic elastase, pancreatin or thermolysin (0.5 mg/ml, respectively) for 5 h at 36°C and boiled. The synthetic glutenin fragments (0.5 mM solution, pH 7.0) were digested with pepsin or pancreatic elastase (0.07 mg/ml) for 5 h at 36°C and boiled.

2.5. Purification of peptides

Separation of peptides in the pepsin–pancreatic elastase digest of wheat gluten was accomplished by reversed-phase HPLC as described previously [1]. Separation of peptides in the digests of synthetic glutenin fragments was accomplished by reversed-phase HPLC on a cyanopropyl silica column (Cosmosil 5CN-R, 4.6×250 mm, Nacalai Tesque, Inc.). The column was developed with a liner gradient between 0 and 40% acetonitrile containing 0.05% TFA at 1 ml/min. The eluate was monitored at 215 nm.

2.6. Opioid activity assay

The opioid activities were measured by the MVD assay as described previously [1]. The mouse vas deferens was suspended with 0.2 g tension in a magnus tube containing a Mg^{2+}-free Krebs-Ringer solution. Electrical stimulation was given to evoke submaximal contraction (20–30 V, 1.2 ms, 0.1 Hz). The contraction was recorded through an isometric transducer (TB-612T, Nihon Koden). This assay was accomplished in the presence of L-leucyl-L-leucine (2 mM), together with bestatin (30 μM), thiorphan (0.3 μM) and captopril (10 μM).

2.7. Amino acid sequence analyses and peptide synthesis

The amino acid sequence of the purified peptide was analyzed by a 477A protein sequencer (Applied Biosystems Inc.). The peptide was synthesized by a PS3 peptide synthesizer (Protein Technologies Inc.). The peptides were purified by reversed-phase HPLC on an ODS column.

3. Results

3.1. Gluten exorphin A5 immunoreactive materials in wheat gluten digests

The polyclonal gluten exorphin A5 antiserum showed cross-reactivities with not only gluten exorphin A5 (Gly–Tyr–Tyr–Pro–Thr) but also Tyr–Tyr–Pro–Thr, while no cross-reactivity with gluten exorphin A4 (Gly–Tyr–Tyr–Pro) (Fig. 1). Gluten exorphin A5 immunoreactive materials in various enzymatic digests of wheat gluten were measured by a competitive ELISA using this antiserum (Table 1). Gluten exorphin A5 immunoreactive materials were recognized in the pepsin–thermolysin digest from which gluten exorphin A5 has been isolated, as expected. Among the gastrointestinal proteases digests, they were found in the pepsin–pancreatin and pepsin–pancreatic elastase digests, but not in the pepsin or pepsin–trypsin–chymotrypsin digests. Especially, the amount of gluten exorphin A5 immunoreactive materials in the pepsin–pancreatic elastase digest was about 4 times as much as that in the pepsin–thermolysin digest. However, the opioid activity in the pepsin–pancreatic elastase digest was almost the equal to that in the pepsin–thermolysin digest on the MVD assay.

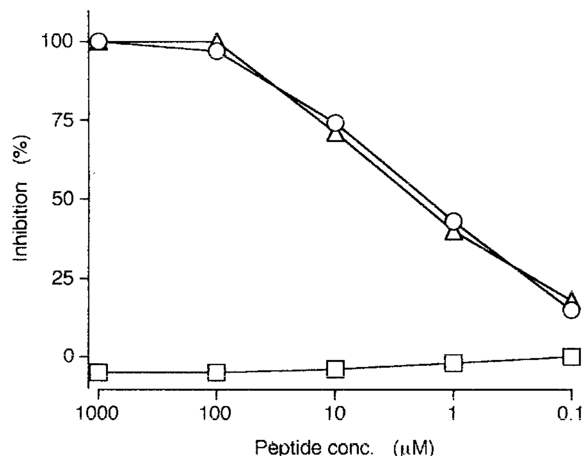

Fig. 1. The standard curves of gluten exorphin A5 and related peptides in a competitive ELISA. The antiserum was diluted at 1000-fold with PBS containing 0.5% Tween 20 and 0.2% NaN_3. Gluten exorphin A5 (Gly–Tyr–Tyr–Pro–Thr), -○-; Tyr–Tyr–Pro–Thr, -△-; gluten exorphin A4 (Gly–Tyr–Tyr–Pro), -□-.

3.2. Isolation of gluten exorphins from the pepsin–pancreatic elastase digest

To quantify individual opioid peptides, the pepsin–pancreatic elastase digest was fractionated by reversed-phase HPLC on an ODS column. The opioid activities were recovered in five fractions (fractions I, II, III, IV and V) (Fig. 2). These fractions were further purified by reversed-phase HPLC on different columns and the structures of purified peptides were analyzed (Table 2). As shown in Table 2, gluten exorphins A5 (fraction II-2), B4 (fraction IV-2), B5 (fraction V-2) and Tyr–Tyr–Pro–Thr lacking the N-terminal Gly of gluten exorphin A5 (fraction I-2) were isolated from the pepsin–pancreatic elastase digest. Although the pure peptide was not obtained from fraction III because of its weak opioid activity, the elution position of fraction III coincided with that of authentic gluten exorphin A4.

The yields of gluten exorphins A and B in the pepsin–elas-

Table 2
Purification of opioid peptides derived from the pepsin–pancreatic elastase digest

Number of opioid fractions (%; acetonitrile)							
ODS[a]		Phenethyl[a]		Cyanopropyl[a]		ODS[b]	
I	(22)	I-1	(20.0)	I-2	(9.0)	I-3	(27.3)
II	(24)	II-1	(24.5)	II-2	(15.0)		
III	(26)	III-1	(26.5)	N.D.[c]			
IV	(30)	IV-1	(31.5)	IV-2	(22.3)		
V	(35)	V-1	(38.8)	V-2	(29.0)		

[a]In the presence of 0.05% TFA.
[b]In the presence of 10 mM potassium-sodium phosphate buffer (pH 7.0).
[c]Not detected.
This table shows the number of opioid active fractions and the acetonitrile concentrations in which they were eluated in various reversed-phase HPLC.

Table 3
Opioid activities, receptor affinities and yields of opioid peptides in the pepsin–pancreatic elastase and the pepsin–thermolysin digests

Peptides		IC50 values (μM)				Yields from the digests	
		Opioid activities[a]		Receptor affinities[a]		Pepsin–elastase	Pepsin–thermolysin
		GPI (μ)	MVD (δ)	[³H]DAGO (μ)	[³H]DADLE (δ)	(μg/g)	(μg/g)
Tyr-Tyr-Pro-Thr		–	800	–	–	500	N.D.[b]
Gly-Tyr-Tyr-Pro-Thr	(Gluten exorphin A5)	1000	60	700	1.5	250	40
Gly-Tyr-Tyr-Pro	(Gluten exorphin A4)	>1000	70	>1000	3.8	N.D.[b]	400
Tyr-Gly-Gly-Trp-Leu	(Gluten exorphin B5)	0.05	0.017	0.045	0.005	0.1	0.1
Tyr-Gly-Gly-Trp	(Gluten exorphin B4)	1.5	3.4	0.17	0.18	3.0	3.0

[a]From reference [1].
[b]Not detected.

tase digest were compared with those in the pepsin–thermolysin digest (Table 3). The yields of gluten exorphins B in both digests were the same, while the yields of gluten exorphins A were different between both digests. The yield of gluten exorphin A5 in the pepsin–pancreatic elastase was about 6 times higher than that in the pepsin–thermolysin digest. Gluten exorphin A4 was almost negligible in the pepsin–pancreatic elastase digest. Moreover, gluten exorphin A5 related peptide, Tyr–Tyr–Pro–Thr which has very weak opioid activity existed in large quantities in the pepsin–elastase digest. This peptide had not been recognized in the pepsin–thermolysin digest.

3.3. *Release of gluten exorphin A5 from synthetic glutenin fragments with pancreatic elastase*

Gluten exorphin A5 is an interspersed repeated sequence which exists 15 times in the primary structure of the high molecular weight glutenin [3]. The amino acids which precede the N-terminal Gly of the gluten exorphin A5 sequence are Gln, Pro, His and Ser, while only Ser exists next to the C-terminal Thr. Therefore, these regions classified into four groups as shown in Table 4. Based on the above regards, we synthesized four typical glutenin fragments (tetradecapeptides) containing gluten exorphin A5 and hydrolyzed them with pepsin and pancreatic elastase. The digests of synthetic glutenin fragments were fractionated by reversed-phase HPLC on a cyanopropyl column and the yield of gluten exorphin A5 in each digest was measured (Table 5). Pepsin did not cleave the peptide bonds at the N- and C-termini of gluten exorphin A5 (data not shown). Gluten exorphin A5 was efficiently released with pancreatic elastase from the Ser-type fragment peptide in which the gluten exorphin A5 sequence is preceded by Ser. In addition, small amounts of gluten exorphins A5 and A4 were found in the digest of the His-type fragment peptide. Tyr–Tyr–Pro–Thr was mainly released from the Pro- and Gln-type fragment peptides.

4. Discussion

The present study demonstrates that gluten exorphins A and B are released by the action of pepsin and pancreatic elastase. Gluten exorphins A and B have been found in the digests which were prepared by the further hydrolysis of the pepsin digest of wheat gluten with microbial neutral proteases such as thermolysin [1]. In the previous paper, we reported that gluten exorphin A5 stimulated the postprandial insulin release after the oral administration in rats and the effect was reversed by co-administration of naloxone [2]. The sequence of this peptide is found 15 times in the primary structure of high molecular weight glutenin [3]. Therefore, it is an interesting problem whether gluten exorphin A5 is released from wheat gluten with gastrointestinal proteases. The similar question has been also indicated about the release of β-casomorphins and their isolation from casein digests with gastrointestinal proteases has been attempted [4–6].

We attempted to detect gluten exorphin A5 immunoreactive materials in the gastrointestinal protease digests of wheat gluten by a competitive ELISA using a polyclonal antibody which recognizes the C-terminus of gluten exorphin A5. The gluten exorphin A5 antiserum showed cross-reactivities with not only gluten exorphin A5 but also Tyr–Tyr–Pro–Thr lacking the N-terminal Gly of gluten exorphin A5. Therefore, it is regarded that the amount of gluten exorphin A5 immunoreactive materials determined in the digest reflects the amount of both gluten exorphin A5 and its analogues with common C-terminus. Gluten exorphin A5 immunoreactive materials were not found in the digests which were hydrolyzed with the combination of pepsin, trypsin and chymotrypsin. This result coincides with the our previous report that gluten exorphin A5 was not isolated from these digests [1]. These were detected in the pepsin–pancreatin and pepsin–pancreatic elastase digests. It is considered that its immunoreactive materials in the pepsin–pancreatin digest were produced by the action of pancreatic elastase, because pancreatic elastase exists in

Table 4
Gluten exorphin A5 sequences in the primary structure of high molecular weight glutenin and typical synthetic glutenin fragments

Gluten exorphin A5 sequences	Synthetic fragments
···GQGQQGYYPTSPQQ··· ···GQGQQGYYPTSLQQ··· ···GQGQQGYYPTSLQQ··· ···GQGQQGYYPTSPQQ··· ···GQGQQGYYPTSLQQ···	GQGQQGYYPTSPQQ
···EQGQPGYYPTSPQQ··· ···GQGQPGYYPTSSQL··· ···GQGQPGYYPTSLQQ··· ···GQGQPGYYPTSPQQ··· ···GQGQPGYYPTSSQQ··· ···GQGQPGYYPTSPLQ··· ···GQGQPGYYPTSPQQ··· ···GQGQPGYYPTSPLQ···	GQGQPGYYPTSPQQ
···GQGQSGYYPTSPQQ···	GQGQSGYYPTSPQQ
···GQGQHGYYPTSPQL···	GQGQHGYYPTSPQL

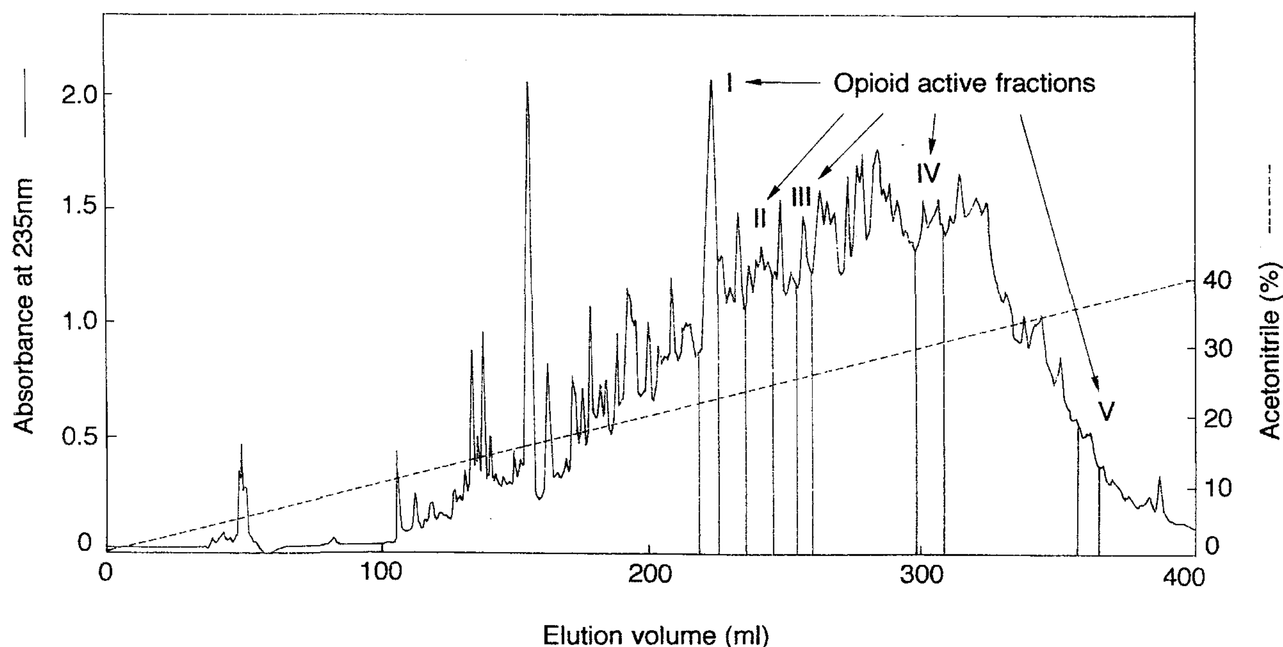

Fig. 2. Reversed-phase HPLC of the pepsin–pancreatin elastase digest on an ODS column. The column was developed using a linear gradient of acetonitrile (0–40%) containing 0.05% TFA at a flow rate of 10 ml/min.

pancreatin. Especially, the amount of gluten exorphin A5 immunoreactive materials in the pepsin–pancreatic elastase digest was larger than that in the pepsin–thermolysin digest.

As shown in Table 2, gluten exorphin A5, B5 and B4 were isolated from the pepsin–pancreatic elastase digest. We could not obtain the pure peptide from fraction III because of the weak opioid activity in this fraction. The retention time of its fraction coincided with that of synthetic gluten exorphin A4 on an ODS column. In addition, a small amount of gluten exorphin A4 was isolated from the pancreatic elastase digest of synthetic glutenin fragment. It is possible that opioid activity in fraction III might be that of gluten exorphin A4.

Pepsin did not hydrolyze the peptide bonds at the N- and C-termini of gluten exorphin A5 of synthetic glutenin fragments. The pretreatment with pepsin might facilitate the release of gluten exorphin A5 with neutral proteases through solubilizing wheat gluten which is insoluble at neutral pH. Under the above condition, gluten exorphin A5 was more efficiently released from the pepsin–pancreatic elastase digest than the pepsin–thermolysin digest. Pancreatic elastase hydrolyzes the peptide bonds at the carboxyl sides of small non-aromatic amino acids such as Gly, Ala and Ser [7]. On the other hand, thermolysin favors hydrolyses of the peptide bond at the amino sides of hydrophobic amino acids [8]. In the primary structure of the high molecular weight glutenin, only Ser exists next to the C-terminal Thr of the gluten exorphin A5 sequence among 15 sites containing the gluten exorphin A5 sequence. The amount of gluten exorphin A5 in the pepsin–pancreatic elastase digest is larger than that in the pepsin–thermolysin digest. As for the release of C-termini of gluten exorphins A, pancreatic elastase exclusively hydrolyzes the Thr–Ser bond, while thermolysin hydrolyzes more efficiently Pro–Thr bond than Thr–Ser bond. Among four series of typical glutenin fragments (tetradecapeptides) containing gluten exorphin A5, gluten exorphin A5 was released quantitatively from the Ser-type glutenin fragment by the action of pancreatic elastase. A small amount of gluten exorphin A5 was also released from the His-type fragment. On the other hand, Tyr–Tyr–Pro–Thr was mainly released from the Pro- and Gln-type fragments. From these results, it is clear that gluten exorphin A5 was released from the very limited regions in its sequence found 15 times in wheat glutenin by the action of pancreatic elastase. If 5 Gln and 8 Pro residues preceding the gluten exorphin A5 sequence in wheat glutenin should be replaced with Ser residue by protein engineering techniques, the release of gluten exorphin A5 with pancreatic elastase might be facilitated.

It has been reported that the oral administration of pepsin digest of wheat gluten influenced the regulation of gastrointestinal motility and hormone release, especially insulin and

Table 5
The yields of YYPT, GYYPT (gluten exorphin A5) and GYYP (gluten exorphin A4) in the pancreatic elastase digests of synthetic glutenin fragments

Synthetic fragments	Yields from the digests (%; mol/mol)		
	YYPT	GYYPT	GYYP
GQGQQGYYPTSPQQ	48	N.D.[a]	N.D.[a]
GQGQPGYYPTSPQQ	85	N.D.[a]	N.D.[a]
GQGQSGYYPTSPQQ	15	84	N.D.[a]
GQGQHGYYPTSPQL	25	3	5

[a]Not detected.

these effects were inhibited by naloxone [9–11]. We isolated gluten exorphins A and B from the pepsin–pancreatic elastase digest of wheat gluten and identified regions which released gluten exorphin A5 by the action of pancreatic elastase in this study. This result suggests that gluten exorphins A and B would be released in vivo by the action of gastrointestinal proteases after the ingestion of wheat gluten. Gluten exorphin A5 shows the stimulatory effect on postprandial insulin release by the oral administration and the effect is reversed by naloxone [2]. Therefore, it is possible that gluten exorphins A5 might be closely related to such a physiological function of the wheat gluten digests reported previously.

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