

Opioid peptides derived from wheat gluten: their isolation and characterization

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Four opioid peptides were isolated from the enzymatic digest of wheat gluten. Their structures were Gly-Tyr-Tyr-Pro-Thr, Gly-Tyr-Tyr-Pro-Tyr-Gly-Gly-Trp-Leu and Tyr-Gly-Gly-Trp, which were named gluten exorphins A5, A4, B5 and B4, respectively. The gluten exorphin A5 sequence was found at 15 sites in the primary structure of the high molecular weight glutenin and was highly specific for δ -receptors. The structure-activity relationships of gluten exorphins A were unique in that the presence of Gly at their N-termini increased their activities. Gluten exorphin B5, which corresponds to [Trp⁴,Leu⁵]enkephalin, showed the most potent activity among these peptides. Its IC_{50} values were 0.05 μ M and 0.017 μ M, respectively, on the GPI and the MVD assays.

Opioid peptide; Wheat; Gluten; Exorphin; Glutenin

1. INTRODUCTION

The presence of opioid peptides in the enzymatic digests of wheat gluten have been recognized. Zioudrou et al. found the opioid activity in the peptic digest of wheat gluten in the assays of both the inhibition of the contraction of the electrically stimulated mouse vas deferens and the inhibition of adenylate cyclase of neuroblastoma X-glioma hybrid cells [1]. Huebner et al. also recognized the presence by the radioreceptor assay [2]. On the other hand, the oral administration of wheat gluten digests prolonged the intestine transit time or increased the plasma insulin level, and the effects were reversed by naloxone [3–5]. However, as for the opioid peptides derived from wheat gluten, no information about their structure and character has been obtained until now. In this paper, we report the isolation and the characterization of four opioid peptides from the enzymatic digest of wheat gluten.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Wheat gluten was obtained from Goodman Fielder Mills Ltd. Pepsin, trypsin and chymotrypsin were from Sigma Chemical Co.

Abbreviations: GPI, guinea pig ileum; MVD, mouse vas deferens; DAGO, [D-Ala²,MePhe⁴,Glycol⁵]enkephalin; DADLE, [D-Ala², D-Leu⁵]enkephalin; EKC, ethylketocyclazocine; ODS, octadecyl silica; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid

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Thermolysin was from Peptide Institute Inc. Other enzymes used were commercial grade. Naloxone was from U.S.P.C., Inc. [³H]DAGO, [³H]DADLE and [³H]EKC were from Amersham. Other reagents used were reagent grade or better.

2.2. Enzymatic digestions of wheat gluten

Wheat gluten (50 mg/ml solution) was digested with pepsin (0.5 mg/ml) in 0.02 N HCl (pH 2.0) for 17 h at 36°C. After the digestion, the pH of the solution was adjusted with 1 N NaOH to 7.0. Then, the solution was boiled and centrifuged. The supernatant was lyophilized. The peptic digest (50 mg/ml solution) was further digested with trypsin or chymotrypsin or thermolysin or other enzymes (0.5 mg/ml, respectively) in distilled water for 5 h at 36°C, boiled and centrifuged.

2.3. Purification of peptides

Separations of peptides in the digests were accomplished by reversed-phase HPLC on an ODS column (Cosmosil 5C₁₈-AR, 20 × 250 mm, Nacalai Tesque Inc.). A 100 mg digest was applied to the column and was eluted with a linear gradient between 0 to 40% acetonitrile containing 0.05% TFA at 10 ml/min. The eluate was monitored at 230 nm. Individual fractions were dried with a centrifugal concentrator and their opioid activities were measured on the MVD assay. The opioid active fractions were purified on an ODS column (Cosmosil 5C₁₈-AR, 4.6 × 150 mm), a phenyl silica column (Cosmosil 5Ph, 4.6 × 250 mm) and cyanopropyl silica column (Cosmosil 5CN-R, 4.6 × 250 mm) from Nacalai Tesque Inc. and a phenetyl silica column (Develosil PhA-5, 4.6 × 250 mm) from Nomura Chemical Inc.. The columns were developed by a linear gradient between 0 to 50% acetonitrile containing 0.05% TFA or 10 mM potassium-sodium phosphate buffer (pH 7) at 1 ml/min. The eluate was monitored at 215 nm to 235 nm. The active fractions were purified on the same ODS column as the first step.

2.4. Opioid activity assays

The measurements of opioid activities were accomplished by the MVD and GPI assays. As for the MVD assay, mouse vas deferens was suspended with 0.2 g tension in a magnus tube containing a Mg²⁺-free Krebs-Ringer solution and stimulated (30 V, 1.2 ms, 0.1 Hz). As for the GPI assay, the guinea pig ileum was suspended with 0.5 g tension in a Krebs-Ringer solution and stimulated (30 V, 0.5 ms, 0.1 Hz).

Contraction was recorded through a isometric transducer. These assays were accomplished in the presence of L-leucyl-L-leucine (2 mM), together with bestatin (30 μ M), thiorphan (0.3 μ M), and captopril (10 μ M). The IC_{50} value is the concentration which inhibits the electrically stimulated muscle contraction by 50%.

The presence of opioid activities in the enzymatic digests of wheat gluten were judged by the MVD assay in the absence and presence of 10^{-6} M naloxone. The activities which were reversed by addition of naloxone and blocked by pretreatment with naloxone were regarded as opioid activities.

2.5. Radioreceptor assay

The radioreceptor assays were performed according to the method of Pert and Snyder [6] in the presence of 1 nM [3 H]DAGO (47.8 Ci/mmol) for the μ -receptors or 1 nM [3 H]DADLE (30.0 Ci/mmol) for the δ -receptors and rat brain membrane. The radioreceptor assay for κ -receptor was performed in the presence of 1 nM [3 H]EKC (24.5 Ci/mmol) and guinea pig cerebellum membrane. All assays were performed in the presence of inhibitors as well as for opioid activity assays. The IC_{50} value is the concentration which inhibits the binding of the labeled ligand by 50%.

2.6. 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 Sam 2 peptide synthesizer (Biosearch Inc.). The peptide was deprotected by the anisol/hydrogen fluoride method and purified by reversed-phase HPLC on an ODS column.

3. RESULTS AND DISCUSSION

Opioid activities in the enzymatic digests of wheat gluten were measured by the MVD assay (Fig. 1). All these digests inhibited the contraction of the electrically stimulated mouse vas deferens. However, these inhibiting activities were composed of the opioid activities which were reversed by naloxone and the non-opioid activities which were not reversed by naloxone. Among

them, the pepsin-thermolysin digest showed the highest opioid activity. Thus, to purify the opioid peptides, we fractionated the pepsin-thermolysin digest by reversed-phase HPLC on an ODS column and assayed the opioid activities of individual fractions on the MVD assay (Fig. 2). Opioid activities were recovered in four fractions (fractions I, II, III and IV) which were eluted at 23%, 25%, 29% and 34% acetonitrile, respectively (Fig. 2). The non-opioid activity, which was not reversed by naloxone, also was eluted at 13% acetonitrile. This non-opioid substance was identified as adenosine from UV, NMR and mass spectra analyses (Data not shown). Authentic adenosine also inhibited the contraction of the electrically stimulated mouse vas deferens and the effect was reversed by its antagonist, theophylline (Data not shown).

We further purified four opioid peptides from the ODS column by reversed-phase HPLC on different columns and obtained four pure peptide fractions, which are illustrated by fraction I-3, II-2, III-3 and IV-5 (Table 1). The yields of individual pure peptides against wheat gluten were $4 \times 10^{-3}\%$ (fraction I-3), $4 \times 10^{-2}\%$ (fraction II-2), $3 \times 10^{-4}\%$ (fraction III-3), and $1 \times 10^{-5}\%$ (fraction IV-5), respectively. The structures of these peptides were analyzed with a protein sequencer and the sequences obtained were Gly-Tyr-Tyr-Pro-Thr (fraction I-3), Gly-Tyr-Tyr-Pro (fraction II-2), Tyr-Gly-Gly-Trp (fraction III-3), and Tyr-Gly-Gly-Trp-Leu (fraction IV-5), which were named gluten exorphins A5, A4, B4 and B5, respectively. Gluten exorphin A5 was an interspersed repeated sequence which was found 15 times in the primary structure of the high molecular weight glutenin [7] (Fig. 3). In addition, Arg-Tyr-Tyr-Pro, Ser-

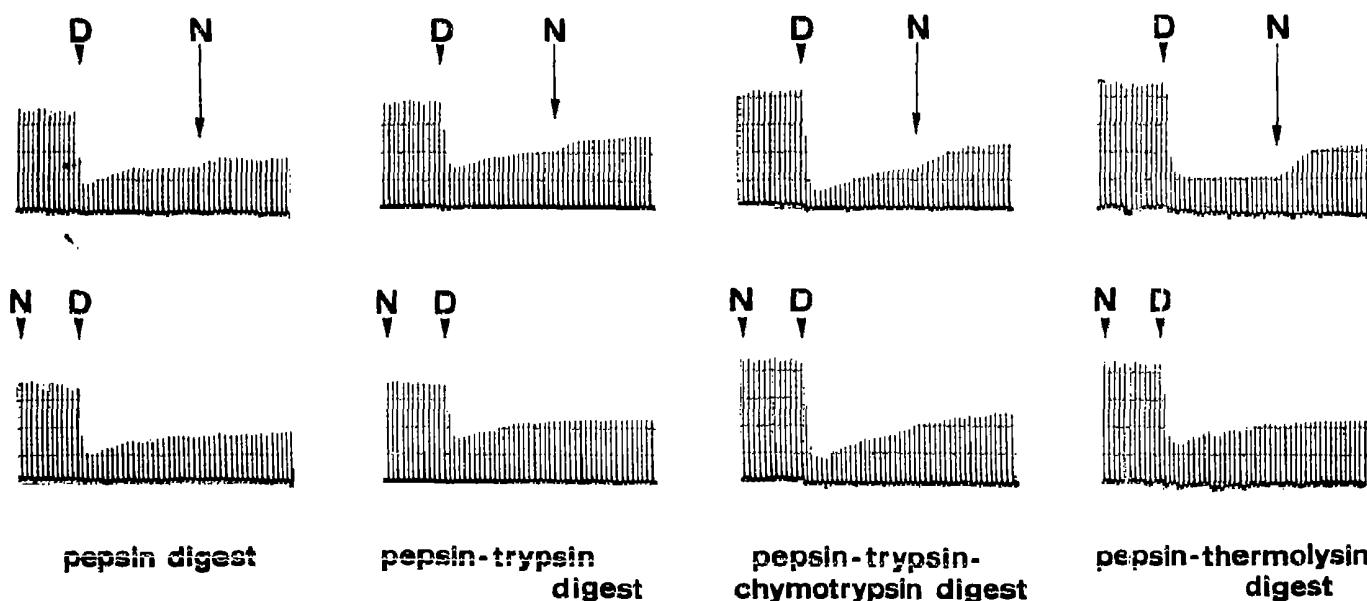


Fig. 1. Effects of enzymatic digests of wheat gluten on the contraction of electrically stimulated mouse vas deferens. Naloxone was added after (above) or before (below) the addition of the digests. D, 10 mg/ml of the enzymatic digest; N, 10^{-6} M naloxone.

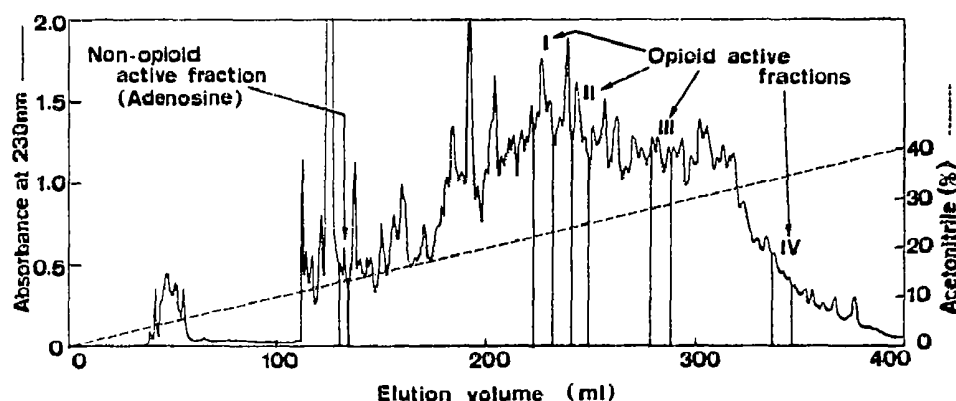


Fig. 2. Reversed-phase HPLC of the pepsin-thermolysin digest on an ODS column.

Tyr-Tyr-Pro, Trp-Tyr-Tyr-Pro, Gly-Tyr-Tyr-Leu and Gly-Tyr-Tyr-Ser, which were homologous to gluten exorphin A4, were also found in this primary structure. On the other hand, gluten exorphins B were not found in the primary structure reported of gliadin or glutenin [7-11]. Wheat gluten is a complex mixture of many different proteins. It is possible that gluten exorphins B are derived from a wheat protein of which the primary structure has not yet been determined. Interestingly, the structure of gluten exorphin B5 corresponds to [Trp¹, Leu⁵]enkephalin. This peptide has been synthesized in the study of the structure-activity relationships of enkephalin derivatives by Schiller et al. [12].

We synthesized these gluten exorphins including their analogues and evaluated their opioid activities by the MVD and the GPI assays and their receptor affinities by the radioreceptor assays (Table II). Gluten exorphins A displayed opioid activities on the MVD assay but not on the GPI assay. Together with the results of radioreceptor assays, it is suggested that gluten exorphins A5 and A4 are δ -selective ligands. These two peptides had no affinity for κ -receptors. The activities of Tyr-Tyr-Pro-Thr and Tyr-Tyr-Pro, which lack an N-terminal Gly of gluten exorphin A5 and A4, were very weak. In the case of the analogues, Arg-Tyr-Tyr-Pro, Ser-Tyr-Tyr-Pro and Trp-Tyr-Tyr-Pro, in which the N-terminal Gly of gluten exorphin A4 is replaced by Arg, Ser or Trp, their activities were less potent than that of gluten exorphin A4. Especially, Trp-Tyr-Tyr-Pro displayed opioid antagonist activity on the MVD assay and its pA₂ value was 3.3. In that an N-terminal Gly increases the activity, the structure-activity relationships of gluten exorphins A are quite different from that of the endogenous opioid peptides. In addition, the structures of gluten exorphins A are also unique in that the two Tyr are continuous. Among the gluten exorphins A and B, the most potent opioid peptide was gluten exorphin B5. Its IC₅₀ values were 0.05 μ M and 0.017 μ M, respectively, on the GPI and the MVD assays. The activity of gluten exorphin B4 was less potent than that of gluten exorphin B5. Especially, this was

remarkable on the MVD assay. It is suggested that Leu⁵ is important for the potency of δ -receptors. In this respect, gluten exorphin B5 is similar to [Leu]enkephalin. Gluten exorphin B5 is the most potent among all the exogenous opioid peptides ever reported. The affinity of gluten exorphin B5 for κ -receptors, however, was remarkably less than those for μ - and δ -receptors (the IC₅₀ value was 30 μ M).

Gluten exorphins A and B obtained from the pepsin-thermolysin digest were not found in other digests of wheat gluten with gastro-intestinal proteases as shown in Fig. 1. However, they were found in digests which were prepared by further hydrolysis of the peptic digest with other microbial neutral proteases derived from *Aspergillus oryzae*, *Bacillus subtilis*, etc. (Data not

Table I

Purification of opioid peptides derived from the pepsin-thermolysin digest. This table shows the number of opioid active fractions which were obtained from fractions I, II, III and IV as shown in Fig. 2 and the acetonitrile concentrations in which they were eluted in various reversed-phase HPLC

Opioid active fraction	Number of opioid active fractions (% acetonitrile)				
	Phenyl ¹	Cyano-propyl ¹	ODS ²	ODS ¹	Phen-ethyl ²
I	I-1 (24.5)	I-2 (19)	I-3 (18)		
II	II-1 (33.5)	II-2 (27)			
III	III-1 (30)	III-2 (26)	III-3 (21)		
IV	IV-1 (36.5)	IV-2 (32.5)	IV-3 (28)	IV-4 (31)	IV-5 (29)

¹ In the presence of 0.05% TFA

² In the presence of 10 mM potassium-sodium phosphate buffer (pH 7)

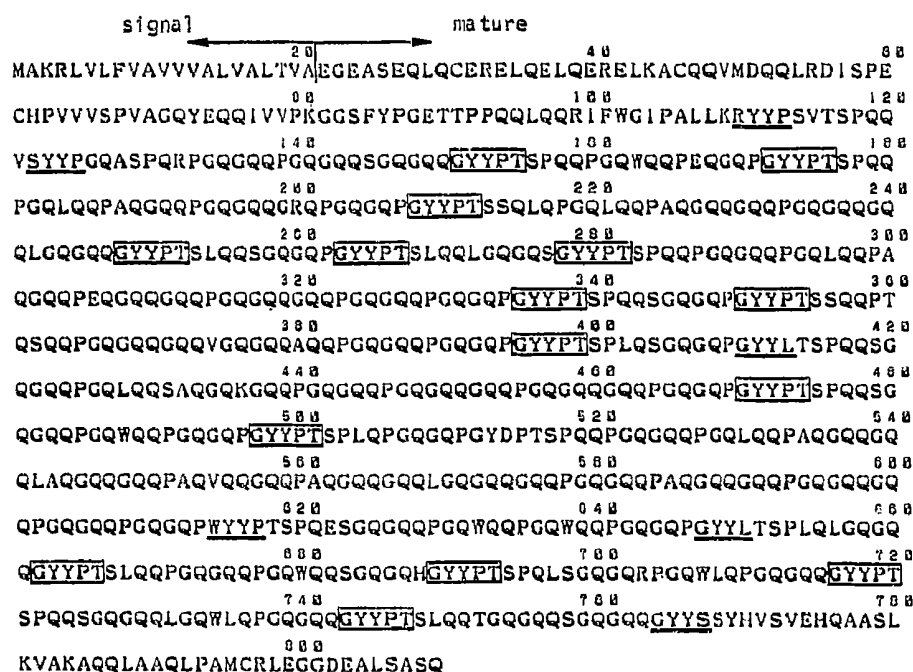


Fig. 3. The primary structure of high molecular weight glutenin reported by Sugiyama et al. [7]. Gluten exorphin A5/4 is boxed. Homologous sequences of gluten exorphin A4 are underlined.

shown). In addition, these peptides were resistant to trypsin and chymotrypsin (Data not shown). These results suggest that, in vivo, the release of gluten exorphins A and B from wheat gluten might be accomplished by the concerted actions of pepsin and entero-

bacterial proteases. A similar example has been reported concerning the release of β -casomorphin from casein, in vivo, where actions of both gastro-intestinal and microbial proteases are involved [13].

It has been reported that the peptic digest of wheat

Table II
Opioid activities and receptor affinities of gluten exorphins A5, A4, B5 and B4 and their analogues

Peptides	Receptor affinities (IC ₅₀)			Opioid activities (IC ₅₀)		
	(³ H)DAGO	(³ H)DADLE	μ/δ	GPI	MVD	μ/δ
	μ (μ M)	δ (μ M)		μ (μ M)	δ (μ M)	
Gly-Tyr-Tyr-Pro-Thr-Ser	1010	5.0	202	>1000	72	-
Gly-Tyr-Tyr-Pro-Thr	700	1.5	467	1000	60	16.7
gluten exorphin A5						
Gly-Tyr-Tyr-Pro	>1000	3.8	-	>1000	70	-
gluten exorphin A4						
Arg-Tyr-Tyr-Pro	(a)	(a)	-	>1000	190	-
Ser-Tyr-Tyr-Pro	>1000	5.6	-	>1000	200	-
Trp-Tyr-Tyr-Pro	700	100	7	(a)	(b)	-
Tyr-Tyr-Pro-Thr	(a)	(a)	-	(a)	800	-
Tyr-Tyr-Pro	>1000	100	-	>1000	1000	-
Gly-Tyr-Tyr	>1000	500	-	>1000	1000	-
Tyr-Gly-Gly-Trp-Leu	0.045	0.005	9	0.05	0.017	2.9
gluten exorphin B5						
Tyr-Gly-Gly-Trp	0.17	0.18	0.94	1.5	3.4	0.44
gluten exorphin B4						
Tyr-Gly-Gly-Phe-Leu	0.037	0.003	12.3	0.04	0.004	10
[Leu]enkephalin						

(a), not determined; (b), this peptide displayed opioid antagonist activity on the MVD assay. (pA₂ value = 3.3)

gluten influenced the regulation of gastro-intestinal motility and hormone release, especially insulin, and that these effects were inhibited by naloxone [3-5]. It is possible that gluten exorphins A5, A4, B5 and B4, which were found in this study, might be related to such a regulation of physiological functions.

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