

Communication

Soymorphins, Novel μ Opioid Peptides Derived from Soy β -Conglycinin β -Subunit, Have Anxiolytic Activities

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Received August 13, 2007; Accepted September 13, 2007; Online Publication, October 7, 2007

[doi:10.1271/bbb.70516]

Based on the amino acid sequence YPFV found in the soy β -conglycinin β -subunit, which is common to an opioid peptide human β -casomorphin-4, peptides YPFV, YPFVV, YPFVVN, and YPFVVNA were synthesized according to their primary structure. On guinea pig ileum (GPI) assay, they showed opioid activity (IC_{50} = 6.0, 9.2 and 13 μ M respectively) more potent than human β -casomorphins, and were named soymorphins-5, -6, and -7, respectively. Their opioid activities on mouse vas deferens (MVD) assay were less potent than on GPI assay, suggesting that they are selective for the μ opioid receptor. Human β -casomorphin-4 and soymorphin-5 were released from the soy 7S fraction (β -conglycinin) by the action of gastrointestinal proteases. Soymorphins-5, -6, and -7 had anxiolytic activities after oral administration at doses of 10–30 mg/kg in the elevated plus-maze test in mice.

Key words: opioid peptide; μ opioid receptor; anxiolytic effect; soy protein; β -conglycinin β -subunit

Opioid is a chemical substance that has morphine-like action. An opioid peptide, bovine β -casomorphin-7 (YPFPGPI), isolated from casein peptone, was the first example of bioactive peptides released from food proteins.¹⁾ We have reported that human β -casomorphin-4 (YPFV) and related peptides derived from human β -casein also have opioid activities, though they are less potent than their bovine counterparts.²⁾ Of three soy β -conglycinin subunits (α , α' , and β),³⁾ the β -subunit has the human β -casomorphin-4 sequence (YPFV). Longer peptides, YPFVV, YPFVVN, and YPFVVNA, were synthesized according to the primary structure of the β -conglycinin β -subunit by the Fmoc strategy, and their opioid activities were tested by guinea pig ileum (GPI) and mouse vas deferens (MVD) assay, as previously described.⁴⁾ All experiments were approved by the Kyoto University Ethics Committee for Animal Research Use.

The peptides were found to have opioid activities on

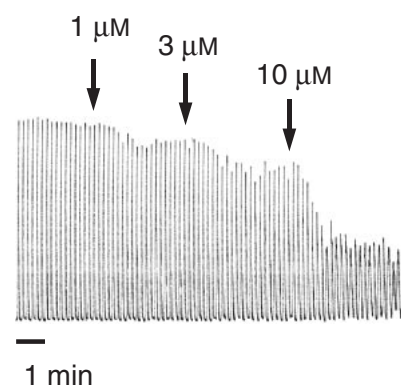


Fig. 1. Typical Opioid Activity of Soymorphin-5 (YPFVV) on Guinea Pig Ileum (GPI) Assay.

Ileum-contracting activity by electrical stimulation was dose-dependently inhibited by soymorphin-5 at doses of 1–10 μ M.

GPI assay. Figure 1 indicates the typical suppressive effect of YPFVV on electrically stimulated contractions on GPI assay. YPFVVN and YPFVVNA also dose-dependently inhibited ileum contractions (data not shown). These results suggest that YPFVV, YPFVVN, and YPFVVNA have opioid activities; they were named soymorphins-5, -6, and -7 respectively. The opioid activities of synthetic peptides derived from the soy β -conglycinin β -subunit are summarized in Table 1. The IC_{50} values of soymorphins-5, -6, and -7 on GPI assay were 6.0, 9.2, and 13 μ M respectively, and their opioid activities were more potent than those of human β -casomorphin-4, -5, and -6 (IC_{50} = 20, 14, and 25 μ M, respectively). There are three types of opioid receptors: μ , δ , and κ .^{5,6)} The opioid activities of soymorphins were larger on GPI assay than those on MVD assay, suggesting that these peptides are selective for the μ -opioid receptor. The affinities for the μ -opioid receptor were 17, 39, and 47 μ M respectively, and the rank order of their affinities for the μ receptor was consistent with that of the opioid activities on GPI assay. Taking all this

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Abbreviations: i.p., intraperitoneal; p.o., per os; GPI, guinea pig ileum; MVD, mouse vas deferens; LAP, leucine aminopeptidase

Table 1. Opioid Activities of Soymorphin-5, -6, and -7 Derived from the Soy β -Conglycinin β -Subunit by Guinea Pig Ileum (GPI) and Mouse Vas Deferens (MVD) Assays, and Their Affinities for the μ -Opioid Receptor

Peptide	Sequence	Opioid activity (IC ₅₀ , μ M)		Affinity (IC ₅₀ , μ M)
		GPI (μ)	MVD (δ)	μ receptor
Soymorphin-5	YPFVV	6.0	50	17
Soymorphin-6	YPFVVN	9.2	32	39
Soymorphin-7	YPFVVNA	13	50	47

μ Receptor binding assay was performed essentially as described previously.^{4,9} The membrane from CHO-K1 cells expressed with human μ opioid receptor (PerkinElmer Life Sciences, Boston, MA) was used with 1 nM of [³H]-DAMGO (American Radiolabeled Chemicals, St. Louis, MO) as a radioligand. Non-specific binding was determined in the presence of 10 μ M DAMGO. The IC₅₀ value is the concentration that inhibited the binding of radioligands by 50%.

Table 2. Enzymatic Release of Soymorphins from the 7S Fraction (β -conglycinin) of Soy Protein and the Peptide Fragment Corresponding to the β -Conglycinin β -Subunit (318–333) by Pancreatic Elastase and Leucine Aminopeptidase (LAP)

Substrate	Peptide yield (mol %)			
	YPFV	YPFVV	YPFVVN	YPFVVNA
VIPAAYPFVVNATSNL	ND	36.5	ND	ND
Soy β -conglycinin	4.8	9.1	ND	ND

ND, Not detected. A synthetic peptide fragment (0.1 mg/ml) corresponding to the soy β -conglycinin β -subunit (318–333) or 10 mg/ml of the 7S fraction (β -conglycinin) was digested with elastase (E/S = 1/20, 4 h) and LAP (E/S = 1/7, 2 h) at 25 °C at pH 7.5. The digest of the fragment peptide was analyzed using an HPLC system and 492 protein sequencer (Applied Biosystems), as previously described.⁹ For the 7S fraction digest, the LC/MS system (Mariner, Applied Biosystems) was used. Separation was performed with a linear gradient of acetonitrile in water containing 0.1% formic acid and 0.01% trifluoroacetic acid (TFA) from 5 to 95% (v/v) for 8 min at a flow rate of 0.2 ml/min using an ODS column, YMC Pack ProC18 (50 mm \times 2.0 mm, YMC., Kyoto, Japan). Peptide fragments were detected by UV absorption spectrometer and electrospray MS in positive ion mode. Soymorphin concentrations in the digest were quantified based on the peak heights of synthetic soymorphins.

together, it was found that soymorphins derived from the soy β -conglycinin β -subunit are more potent μ -opioid peptides than are human β -casomorphins.

Opioid peptides having a Tyr-Pro-aromatic amino acid sequence are selective for the μ receptor. This is consistent with the fact that soymorphins containing the YPF sequence are μ -selective. Casomorphins (*e.g.*, human β -casomorphin-7, YPFVEPI),² hemorphin (YPWT) derived from hemoglobin,⁷ and endomorphin-1 and 2 (YPWF-NH₂ and YPFF-NH₂ respectively)⁵ containing the Tyr-Pro-aromatic amino acid sequence in these molecules, are also μ -selective opioid peptides, of animal origin. In contrast, opioid peptides of plant origin, such as gluten exorphin^{4,8} and rubiscolin,^{9,10} with the Tyr-Pro-non-aromatic amino acid sequence, are selective for the δ receptor. Soymorphins were the first μ -selective opioid peptides of plant origin to be identified.

To determine whether soymorphins are released from the soy β -conglycinin β -subunit containing the amino acid sequence of YPFV (human β -casomorphin-4 or soymorphin-4), a model peptide corresponding to the β -conglycinin β -subunit (318–333) was synthesized and digested with gastrointestinal proteases. Ala⁰-soymorphin-5 (AYPFVV), which is released from the model peptide after digestion of pancreatic elastase (Elastin Products, Owensville, MO) has less potent opioid activity than soymorphin-5. Ala⁰-soymorphin-5 was quantitatively converted to soymorphin-5 by digestion of leucineamino peptidase (LAP, Sigma-Aldrich, St. Louis, MO) with an overall yield of 36.5% (Table 2), but, soymorphins-4, -6, and -7 were not released under

these conditions. We also digested the soy 7S fraction (β -conglycinin), which was kindly provided by Fuji Oil Co., Ltd. (Osaka Japan), with pancreatic elastase followed by LAP (Table 2). Human β -casomorphin-4 and soymorphin-5 were detected at 4.8 and 9.1%. They might have been produced in the gastrointestinal tract by digestive enzymes.

It is known that opioids are associated with emotional behavior.¹¹ Hence we tested whether soymorphins have anxiolytic activities using the elevated plus-maze test in mice, as previously described.^{11,12} Intraperitoneally (*i.p.*) administered soymorphins-5, -6, and -7 at doses of 3–10 mg/kg increased the percentage (%) of time spent in the open arms (Fig. 2a–c), indicating that these peptides have anxiolytic activities. The dose-response curves for soymorphin-5 and -6 were bell-shaped. On the other hand, YPFV (human β -casomorphin-4 or soymorphin-4) and YPFVE (human β -casomorphin-5) did not show an anxiolytic effect at doses of 10–100 mg/kg after *i.p.* administration (data not shown). For anxiolytic activity, Val⁵ in soymorphin-5 might be better than Glu⁵ in human β -casomorphin-5.

Orally administered soymorphins-5, -6, and -7 also increased the percentage of time spent in open arms (Fig. 2d–f), suggesting that they are orally active peptides having anxiolytic activities. This suggests that at least a part of soymorphin might be absorbed intact. The minimum effective doses for anxiolytic activities of soymorphins-5 and 6 after oral administration (10 mg/kg) were 3-fold higher than those after *i.p.* injection (3 mg/kg). In contrast, the minimum effective dose of soymorphin-7 (30 mg/kg) after oral administration was

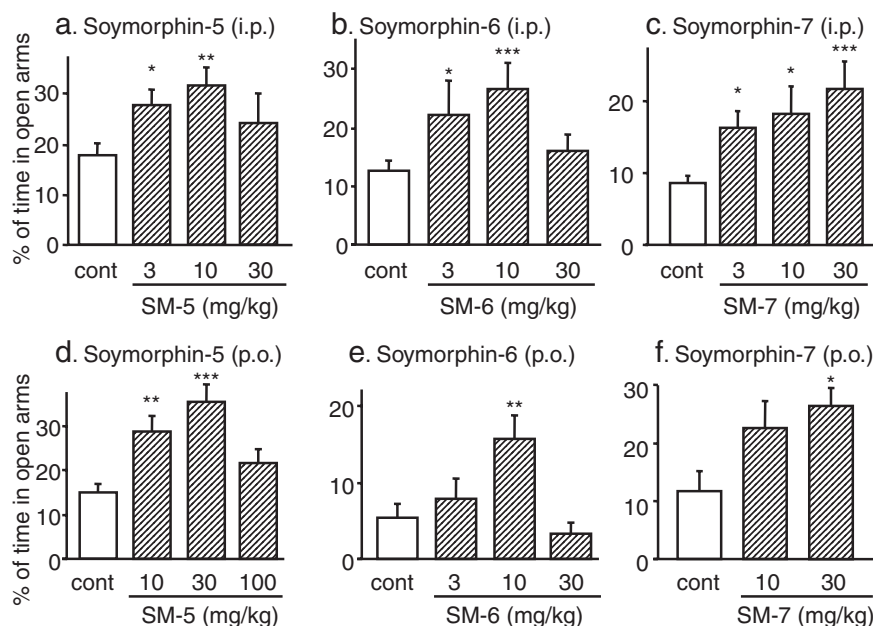


Fig. 2. Anxiolytic Activities of Soymorphins (SM)-5, -6, and -7 after i.p. and Oral Administration at Doses of 3–100 mg/kg in the Elevated Plus-Maze Test in Male ddY Mice.

Soymorphin-5 (a,d), -6 (b,e), or -7 (c,f) was intraperitoneally (i.p., a–c) or orally (p.o., d–f) administered 30 min before the tests. Values are presented as means \pm SEM (a–c: $n = 11$ –21, d–f: $n = 7$ –18). Analysis of variance (ANOVA) followed by Fisher's tests were used to assess differences among groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared with the saline control group.

10-fold higher than that after i.p. injection (3 mg/kg). This might be explained by the larger molecular size of soymorphin-7, possibly decreasing the ability to penetrate the gut-blood barrier. In addition, soymorphins-5, -6, and -7 did not change total entry in the elevated plus-maze test (data not shown), suggesting that soymorphins did not change locomotor activity under our experimental conditions. Soymorphins-5, -6, and -7 did not show any anti-nociceptive effect under these conditions (data not shown). Therefore, the dose requirement for anxiolytic activity is different from analgesia.

We have reported that rubiscoin-6, a δ -opioid peptide derived from a large subunit of spinach D-ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), has anxiolytic activity at a dose of 100 mg/kg after oral administration, and that its anxiolytic activity was mediated through σ_1 and dopamine D₁ receptors.¹²⁾ Soymorphin-5-induced anxiolytic activity was not inhibited by a D₁ receptor antagonist SCH23390 (data not shown), suggesting that the mechanism of anxiolytic activity of μ opioid soymorphin-5 might be different from that of δ opioid rubiscoin-6. It has been reported that the stress-induced increase in norepinephrine release in the CNS was attenuated by μ agonists such as morphine and β -endorphin.¹³⁾ Further investigation should reveal the mechanism of anxiolytic activity of μ -opioid soymorphins.

Many bioactive peptides derived from soy protein have been identified, and we have also reported an immunostimulating peptide soymetide derived from soy β -conglycinin.¹⁴⁾ It is well-known that soy has many beneficial effects in preventing lifestyle-related diseases

(e.g., cholesterol- and triglyceride-lowering effects),^{14,15)} some of which have been attributed to soy proteins and peptides.¹⁴⁾ Recently, it was reported that the intake of soy protein and its digestion suppress stress levels in humans.¹⁶⁾ The anxiolytic effects of soymorphins derived from soy protein might contribute to the stress-lowering effects of soy. To the best of our knowledge, anxiolytic peptides, "soymorphins," are the first example of μ -opioid peptides derived from soy protein.

In conclusion, soymorphins-5, -6, and -7, corresponding to the amino acid sequence in the β -conglycinin β -subunit of soy protein, have opioid activities and are selective for the μ -opioid receptor in GPI and MVD assays. We also identified enzymatic condition for releasing soymorphins from soy protein by proteases present in the gastrointestinal tract. Soymorphins showed anxiolytic-like activities in the elevated plus-maze test in mice, which were present when they were administered orally (10–30 mg/kg) or i.p. (3 mg/kg).

Acknowledgments

This work was supported in part by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, to MY and KO, and by a PROBRAIN grant from the Bio-Oriented Technology Research Advancement Institution, to MY.

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