# Rubiscolin, a δ selective opioid peptide derived from plant Rubisco

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**Abstract** We found that the sequences YPLDL and YPLDLF in the large subunit of spinach D-ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) met the structure YP-aliphatic amino acid which might have opioid activity. We then synthesized these peptides to test their opioid activity. The $IC_{50}$ of these peptides in mouse vas deferens assay were 51.0 μM and 24.4 μM, respectively, and those in δ receptor binding assay using [$^3$H]deltorphin II as radioligand were 2.09 μM and 0.93 μM, respectively. Both peptides were selective for δ receptor. We named them rubiscolin-5 and -6, respectively. Rubiscolin-5 and -6 have antinociceptive activity in mice after i.c.v. or oral administration. The enzymatic conditions to release rubiscolin were investigated using both spinach Rubisco and synthetic fragment peptides. This is the first example of bioactive peptides derived from plant Rubisco. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

*Key words:* δ opioid peptide; Mouse vas deferens; Guinea pig ileum; Antinociception; Receptor binding; Rubisco

## 1. Introduction

Rubisco, (D-ribulose-1,5-bisphosphate carboxylase/oxygenase; EC 4.1.1.39), is the key enzyme for carbon dioxide fixation and photorespiration. This enzyme is responsible for food production for the entire biosphere. Because of its abundance (about 30–50% of soluble protein in green leaves of plants), the enzyme itself serves as food. Many kinds of bioactive peptides have been isolated from enzymatic digests of food proteins. In this study, we investigated whether any bioactive peptides are derived from Rubisco.

It was well established that opioid peptides containing YPX sequence in these molecules usually have aromatic amino acids X; for example, X is Phe in β-casomorphin and endomorphin-2, while in hemorphin and endomorphin-1, X is Trp [1–3]. However, accumulating evidence indicates that aliphatic amino acids X in YPX sequence also contribute to onset of opioid activity, as in the case of gluten exorphin A5 (GYYPT) [4], gluten exorphin C (YPISL) [5], Tyr-MIF-1 [6], and neocasomorphin (YPVEPF) [7]. Given all this, we hypothesize that aliphatic X in addition to aromatic ones, can also induce opioid activity. To sustain this hypothesis, we searched for the relevant sequence in spinach Rubisco large subunit and noticed that sequences YPLDL (residues 103–107) and YPLDLF (residues 103–108) would meet the above-mentioned requirement. To scrutinize their properties in detail, we synthesized these peptides, and investigated their opioid nature as well as the condition leading to their release by intestinal enzymatic digestions.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Fmoc-amino acids and Alko resins were from Watanabe Chemicals Co. Ltd, Japan. Naltrindole was from RBI (Natick, MA, USA). [$^3$H][D-Ala$^2$, MePhe$^4$, Glycol$^5$]enkephalin (DAMGO), [$^3$H]deltorphin II (Delt II) and [$^3$H][D-Pen$^2$, D-Pen$^5$]enkephalin (DPDPE) were from NEN (Boston, MA, USA). Spinach Rubisco, pepsin, chymotrypsin, leucine aminopeptidase (LAP) and protease inhibitor cocktail (product number P8340) were from Sigma. Elastase and naloxone hydrochloride were from Wakenyaku Co., Ltd, Japan.

### 2.2. Animals

Male Hartley guinea pigs for guinea pig ileum (GPI) assay, male Wistar rats for brain membrane preparation, retired male ICR mice for mouse vas deferens (MVD) assay, and male ddy mice for antinociceptive test were purchased from Shimizu Experimental Animals (Kyoto, Japan). They were housed for at least 2 days under standard conditions ($23 \pm 1$°C, $55 \pm 5\%$ humidity, light–dark cycle with light on between 7.00 and 19.00 h) with free access to food and water. All procedures in this study were performed in accordance with the guidelines for the care and use of laboratory animals of Kyoto University.

### 2.3. Peptide synthesis and purification

Peptides (YPLDL, YPLDLF and ICYVAYPLDLFEEG corresponding to residues 98–111 of spinach Rubisco large subunit) were synthesized on PS3 peptide synthesizer (Protein Technologies) by Fmoc strategy. Synthetic peptides were purified by HPLC on an octadecyl silica (ODS) column (Cosmosil 5C18-AR, $20 \times 250$ mm, Nacalai Tesque). The synthetic peptide fragment for enzymatic hydrolysis was further purified on a phenethyl silica column (Cosmosil 5PE-MS, $10 \times 250$ mm, Nacalai Tesque). The column was developed with a linear gradient of acetonitrile (0–40%/40 min) containing 0.1% TFA at flow rates of 10 ml/min for ODS column and 3 ml/min for phenethyl silica column.

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*Abbreviations:* Rubisco, D-ribulose-1,5-bisphosphate carboxylase/oxygenase; MVD, mouse vas deferens; GPI, guinea pig ileum; LC-Mass, liquid chromatography coupled with mass spectrography; DAMGO, [D-Ala$^2$, MePhe$^4$, Glycol$^5$]enkephalin; DPDPE, [D-Pen$^2$, D-Pen$^5$]enkephalin; Delt II, deltorphin II; AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride; i.C.V., intracerebroven tricularly; p.o., per oral
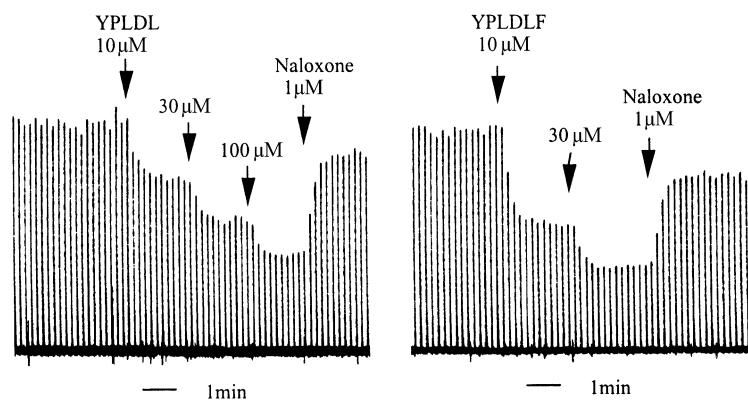
Fig. 1. Typical suppressive effect of rubiscolin-5 and -6 on field-stimulated contraction in MVD assay.

### 2.4. Opioid activity assays

Opioid activity was measured by MVD assay and GPI assay as described previously [4]. Antinociceptive effect was tested in mice by tail pinch method according to [8].

### 2.5. Radioreceptor assay

Receptor binding assay was accomplished essentially as described [4]. For μ receptor binding assay, rat brain membrane was used at a final protein concentration of 1 mg/ml with 1 nM [3H]DAMGO as radioligand. For δ receptor binding assay, one set of experiment was done with rat brain membrane using 1 nM $\delta_2$ agonist [3H]Delt II as radioligand, and another set with membrane (final protein concentration 60 μg/ml) made from CHO cells stably expressing human δ opioid receptor using 1 nM $\delta_1$ agonist [3H]DPDPE as radioligand. The transfected CHO cell was cultured and membrane was prepared as described [9]. Non-specific binding was determined in the presence of 10 μM naloxone. All assays were performed in the presence of protease inhibitor cocktail containing 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) 1 mM, aprotinin 0.8 μM, leupeptin 22 μM, bestatin 40 μM, pepstatin A 15 μM, and E-64 14 μM. The $IC_{50}$ value is the concentration that inhibits the binding of radioligands by 50%.

### 2.6. Enzymatic digestion of spinach Rubisco and synthetic peptide fragment

50 mg of spinach Rubisco at a concentration of 10 mg/ml was digested by pepsin (E/S = 1/50, w/w) for 4 h at 37°C. The reaction was stopped by adjusting the pH to 8.0 with 1 N NaOH. Then, half of the digest was further hydrolyzed by LAP for 8 h at 37°C (E/S = 1/50, w/w), and the hydrolysis was stopped by boiling. The digests of spinach Rubisco were fractionated by HPLC on an ODS column (Cosmosil 5C18-AR, 20×250 mm, Nacalai Tesque) with a linear gradient of acetonitrile (0–50%/50 min) containing 0.1% TFA at a flow rate of 10 ml/min, and dried with a centrifugal concentrator. Potential fractions judged by their retention time relative to those of standard YPLDLF and YPLDL were further purified on a phenethyl silica column (Cosmosil 5PE-MS, 4.6×250 mm, Nacalai Tesque) with a linear gradient of acetonitrile (0–50%/50 min) containing 0.1% TFA at flow rate of 1 ml/min. The potential fractions were further purified on a cyanopropyl silica column (Cosmosil 5CN-R, 4.6×250 mm, Nacalai Tesque), dried on centrifugal concentrator and analyzed by Protein Sequencer (Type 492, Applied Biosystems), or confirmed by liquid chromatography coupled with mass spectrography (LC-Mass, Mariner, Applied Biosystems). The peptide ICYVAYPLDLFEEG corresponding to the residues 98–111 in the large subunit of spinach Rubisco was digested at a concentration of 2 mg/ml (200 μl/experi-

ment) with pepsin, elastase and chymotrypsin (E/S = 1/20, w/w) for 2 h at 37°C. Half of the digests was further digested by LAP (E/S = 1/20, w/w) for 1 h at 37°C. The pepsin digest was neutralized by NaOH. Finally, the reactions were stopped by boiling. The digests of synthetic peptide fragment were applied onto LC-Mass directly. The yields of individual peptides were calculated by the area of absorbance at 280 nm in HPLC or LC-Mass charts.

### 2.7. Data analyses

Data from GPI assay, MVD assay, receptor binding assay, and antinociceptive test were expressed as means ± S.E.M. Comparisons between two groups were carried out by Student's *t*-test. For all statistical analyses, the differences were considered significant at $P < 0.05$.

## 3. Results

### 3.1. Opioid activities of synthetic YPLDL and YPLDLF

To test whether the sequences YPLDL and YPLDLF have opioid activity, we synthesized them and applied them to in vitro biological assays. In MVD assay, YPLDL and YPLDLF exhibited typical suppressive activity on field-stimulated contraction (Fig. 1). Naloxone reversed their effects, indicating opioid activity of these peptides. The $IC_{50}$ of YPLDL and YPLDLF in MVD assay were 51.0 μM and 24.4 μM, respectively, while those in GPI assay were 1110 μM and 748 μM, respectively. Accordingly, both peptides are characterized as δ selective opioid agonists. The selectivity toward δ against μ in terms of $IC_{50}$ ratio in GPI and MVD assays were 21.8 for YPLDL and 30.7 for YPLDLF. In δ receptor binding assay, YPLDL showed $IC_{50}$ of 1.97 μM in replacing [3H]DPDPE and 2.09 μM in replacing [3H]Delt II, with the $IC_{50}$ of YPLDLF being 0.90 μM and 0.93 μM, respectively (Table 1). There is not much difference in their δ receptor affinity irrespective of $\delta_1$ ([3H]DPDPE) or $\delta_2$ ([3H]Delt II) agonist being used as radioligand. The $IC_{50}$ of YPLDL in μ receptor binding assay using [3H]DAMGO as radioligand was 1085 μM, and that for YPLDLF was more than 2000 μM. In accordance with the results from MVD and GPI assay, both

Table 1
Opioid activities and receptor affinities of rubiscolin-5 and -6

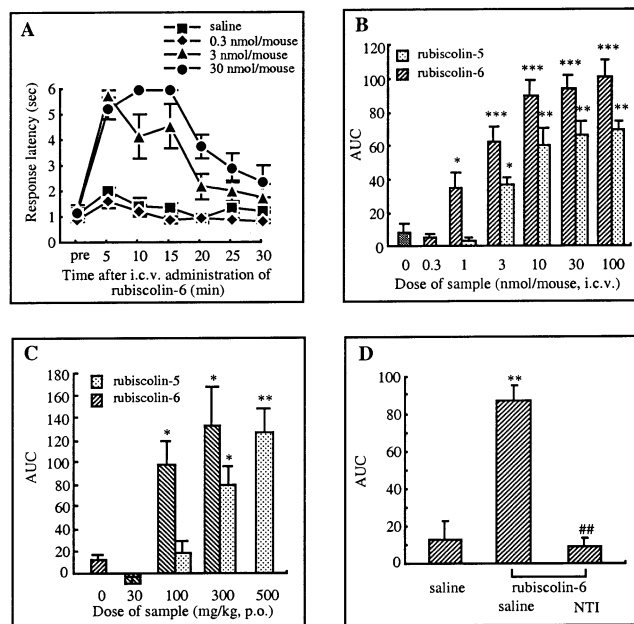| Peptides | Opioid activities $IC_{50}$ (μM) | | GPI/MVB | Receptor affinities $IC_{50}$ (μM) | | |
| --- | --- | --- | --- | --- | --- | --- |
| | GPI | MVD | | [3H]DAMGO | [3H]Delt II | [3H]DPDPE |
| YPLDL (rubiscolin-5) | 1110 ± 71 | 51.0 ± 6.6 | 21.8 | 1085 ± 165 | 2.09 ± 0.06 | 1.97 ± 0.30 |
| YPLDLF (rubiscolin-6) | 748 ± 207 | 24.4 ± 3.6 | 30.7 | > 2000 | 0.93 ± 0.04 | 0.90 ± 0.29 |

Fig. 2. Antinociceptive effects of rubiscolin-5 and -6 in tail pinch test in mice. A: Time course of rubiscolin-6 after i.c.v. administration. B: Dose response of rubiscolin-5 and -6 after i.c.v. administration. C: Dose response of rubiscolin-5 and -6 after p.o. administration. D: Effect of δ opioid antagonist naltrindole (NTI, 1 mg/kg, s.c.) on the antinociceptive effect of rubiscolin-6 (35 nmol/mouse, i.c.v.). NTI was given 10 min before rubiscolin-6 administration (i.c.v.). In B, C and D, the AUC of response latency versus time was calculated for each mouse. Results are expressed as mean ± S.E.M., with $n = 5$ or 6 for each group. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ versus saline control. $##P < 0.01$ versus saline (s.c) followed by rubiscolin-6 (i.c.v.).
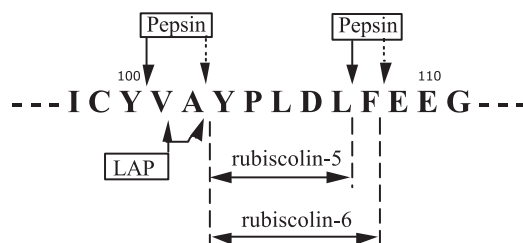


Fig. 3. Cleavage sites in spinach Rubisco large subunit by gastrointestinal proteases leading to the release of rubiscolin-5 and rubiscolin-6. Solid arrows indicate main sites, while dashed arrows indicate minor sites.

and 1 nmol/mouse, respectively. Fig. 2A shows the representative time course of the antinociceptive effect of rubiscolin-6, which lasts for nearly 30 min. The dose response is given in Fig. 2B after calculating the area under curve (AUC) in the time course graph. Both peptides also exhibited antinociceptive effect after oral administration at higher doses (Fig. 2C). The results indicate that rubiscolin-6 is nearly three times more potent than rubiscolin-5. The antinociceptive effect was mediated by δ opioid receptor because a selective δ opioid antagonist naltrindole blocked antinociception induced by rubiscolin-6 (Fig. 2D).

### 3.3. Enzymatic release of rubiscolins from spinach Rubisco and synthetic precursor

After confirming their typical δ opioid activities, we then investigated whether rubiscolin-5 and -6 could be released from Rubisco by gastrointestinal enzymatic digestion. Our results evidenced that rubiscolin-5 could be released from spinach Rubisco after pepsin digestion with a yield of 0.17% mol per mol Rubisco large subunit. Subsequent LAP digestion of the pepsin digest of spinach Rubisco improved the yield of rubiscolin-5 to 5.0% (Table 2). Under the same condition, rubiscolin-6 was not detected. Digestion of synthetic peptide fragment ICYVAYPLDLFEEG might allow a better understanding of the sites and extent of enzymatic cleavage. Therefore, we synthesized this fragment, which corresponds to residues 98–111 of spinach Rubisco large subunit. Pepsin digestion of this peptide fragment can release both YPLDL and YPLDLF with the yield of 0.30% and 0.04%, respectively. The predominant product by pepsin digestion is VAYPLDL, suggesting that the peptide bond between Tyr100 and Val 101, and that between Leu107 and Phe108 are readily cleaved by pepsin (Fig. 3).

Coupled with the finding that trace amounts of VAYPLDLF and YPLDLF were also released, it is conceivable that pepsin can also cleave the peptide bond between Ala102 and Tyr103, and that between Phe108 and Glu109 (Fig. 3). Subsequent LAP digestion greatly improved the yield of YPLDL up to 40% (Table 2), presumably as a result of LAP-induced removal of Val and Ala from VAYPLDL, albeit no cleavage of Y-P bond. However, the yield of YPLDLF was slightly decreased. It is possible that some other contaminating enzyme in LAP might be responsible for this decrease, because it can not be explained by the specificity of LAP as described above. The enzymatic cleavage sites for pepsin and LAP are given in Fig. 3. We also hydrolyzed the synthetic fragment by pancreatic elastase and chymotrypsin. Nevertheless, neither YPLDL/YPLDLF nor amino-/car-

peptides were found rather weak as μ agonists. The homology of Rubisco large subunits is relatively high among many species, besides the sequence YPLDLF being preserved in most plant species. Now that properties of both peptides are characterized, we named them rubiscolin-5 and rubiscolin-6, respectively.

### 3.2. The antinociceptive effect of rubiscolin-5 and rubiscolin-6

After demonstrating the δ opioid activity of rubiscolin-5 and -6 in in vitro bioassays, we proceeded to investigate their effects in vivo by determining their antinociceptive effects in mice using the tail pinch method. Rubiscolin-5 and rubiscolin-6 gave significant antinociceptive effect after i.c.v. administration at minimum doses of 3 nmol/mouse

Table 2
Enzymatic release of rubiscolin-5 and -6 from spinach Rubisco or from peptide fragment corresponding to spinach Rubisco large subunit (98–111)

| Substrate(s) | Enzyme(s) | Peptide yield (mol%) | |
|---|---|---|---|
| | | YPLDL | YPLDLF |
| Spinach Rubisco | Pepsin 4 h | 0.17 | ND |
| | Pepsin 4 h, LAP 8 h | 5.0 | ND |
| ICYVAYPLDLFEEG | Pepsin 2 h | 0.30 | 0.04 |
| | Pepsin 2 h, LAP 1 h | 40 | 0.02 |

The yields from spinach Rubisco were expressed as (mol peptide/mol large subunit, %). ND means not detected.

boxyl-terminally elongated ones were detected (data not shown).

## 4. Discussion

From the digests of food proteins, many bioactive peptides have been isolated, such as opioid and opioid antagonist peptides [1,3–5,7,10–18], vasorelaxing or vasoconstricting peptides [19,20], inhibitory peptides of angiotensin-converting enzyme [21–23] and immunostimulating peptides [24–26]. Because of the abundance of Rubisco in plant green leaves including some vegetables, Rubisco should be a protein source not only for animals but also for human being. By analyzing the amino acid sequence of Rubisco large subunit, we selected two peptide fragments (rubiscolin-5 and rubiscolin-6) which could be released by enzymatic digestion and express affinities to opioid receptors. In the present study we isolated them from intestinal enzymatic digests of Rubisco or synthetic intermediary peptide. The yield of rubiscolin-5 is quite high in the digests of both spinach Rubisco and synthetic fragment, and it might have some physiological significance. Although we could not detect rubiscolin-6 in the pepsin-LAP digest of spinach Rubisco, it could be released by the same enzyme from a synthetic intermediary peptide. We cannot rule out the possibility that rubiscolin-6 be released under in vivo condition. This is the first example that bioactive peptides were obtained from Rubisco by intestinal enzymatic digestion.

Considering the structure of rubiscolin-5 and -6 which share the common sequence YPL- therein, our results further favored our hypothesis that sequence YPX comprising aliphatic X besides aromatic X can also induce opioid activity.

Previously, we isolated a δ opioid peptide gluten exorphin A5 (GYYPT) from digest of wheat gluten [4]. Its $IC_{50}$ in MVD assay was 60 µM, being weaker than our newly isolated rubiscolin-5 and -6. The selectivity of gluten exorphin A5 toward δ against µ in terms of $IC_{50}$ ratio in GPI and MVD assays was 16.7, while those of rubiscolin-5 and -6 were 21.8 and 30.7, respectively. Similarly, µ/δ selectivity in receptor binding assays was 467 for gluten exorphin A5, whereas those of rubiscolin-5 and -6 were >500 and >2000, respectively. Gluten exorphin B5 (YGGWL), another δ opioid peptide derived from wheat gluten, was a very potent δ agonist ($IC_{50} = 0.017$ µM in MVD assay); however, its selectivity µ/δ (2.9 in GPI/MVD, 9.0 in receptor binding) [4] was less than those of rubiscolin-5 and rubiscolin-6. Thus, to our best knowledge, rubiscolins are the most selective δ opioid peptides derived from food protein, so far reported.

When applied i.c.v., rubiscolins showed an antinociceptive effect that is typical for opioid. Interestingly, both peptides induced antinociception after oral administration, which never occurs for endogenous opioid peptides. The reason might be interpreted by the concept that endogenous opioid peptides as well as some exogenous opioid peptides such as gluten exorphin B5 (which has the similar structure with enkephalins) are rather vulnerable to endogenous peptidase, while rubiscolins would be more stable due to the sequence YP- in their molecules. Although gluten exorphin A5 also possesses the sequence YP-, it failed in inducing antinociception at a dose of 300 mg/kg after oral administration [27]. In contrast, however, significant antinociception was induced by rubiscolin-5 at the same dose, and rubiscolin-6 induced antinociception at

even a lower dose of 100 mg/kg. This result is consistent with the finding that rubiscolin-5 and -6 are more potent than gluten exorphin A5.

It is worthy to note that food protein derived opioid peptides of animal origin such as hemorphin and β-casomorphin are mainly µ selective, while those of plant origin such as gluten exorphin A and B, as well as our newly isolated rubiscolin are mainly δ selective. Despite of their biological significance being unclear, rubiscolins might appear on the earth much earlier than did animal opioid receptors and endogenous opioid peptides. In this context, their effect on plants themselves and insects may pose another intriguing question for us.

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