Introduction of DPR, an Enterostatin Fragment Peptide, into Soybean β-Conglycinin α’ Subunit by Site-directed Mutagenesis

Yasuyuki Takenaka,¹,* Naomi Doyama,¹ Nobuyuki Maruyama,² Shigeru Utsumi,² and Masaaki Yosikawa¹

¹Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Uji, Kyoto 611-0011, Japan
²Division of Agronomy and Horticultural Science, Graduate School of Agriculture, Kyoto University, Uji, Kyoto 611-0011, Japan

Received August 12, 2003; Accepted September 30, 2003

DPR, a fragment peptide of enterostatin (VPDPR) having hypocholesterolemic activity, was introduced into the three homologous sites, EPR, DYR, and DPI, in the soybean β-conglycinin α’ subunit by site-directed mutagenesis. The modified β-conglycinin was expressed in Escherichia coli and recovered in the soluble fraction. After purification on ion-exchange HPLC, the modified β-conglycinin was digested by trypsin to release integrated DPR. The yield of DPR from 1 mole of the modified β-conglycinin was 1.2 mole.

Key words: enterostatin fragment peptide; hypocholesterolemic activity; β-conglycinin; soybean

Enterostatin (VPDPR), which is released from the amino terminus of procolipase on its conversion to colipase, inhibits food intake in animals fed a high-fat diet.¹⁻⁰ Recently, we found that VPDPR and its fragment peptide DPR had hypocholesterolemic effects after oral administration in mice fed a high cholesterol-cholic acid diet, with the effect of DPR being stronger than that of VPDPR.⁹ In order to exploit this hypocholesterolemic effect of DPR, we incorporated the DPR sequence into three homologous sites, EPR, DYR, and DPI, which the cDNA encoding the β-conglycinin α’ subunit, was used to transform the host strain, HMS174(DE3), which was grown as 2.8L Luria-Bertani (pH 7.0) cultures in the presence of 50 µg/ml ampicillin at 37°C, and induced with 1 mM IPTG for 48 h at 20°C. The induced cells were harvested by centrifugation. Portions of the cells were boiled in SDS sample buffer (62.5 mM sodium phosphate, pH 7.6, 0.2 M NaCl, 10 mM 2-mercaptoethanol), and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with 11% acrylamide gels. Both the wild-type and DPR-expressing subunits were recognized as single bands by anti-recombinant β-conglycinin serum (data not shown).

The expression plasmid pECα’, or the mutated pECα’ encoding the DPR-introduced α’ subunit, was used to transform E. coli HMS174(DE3) (Novagen) was used as the host. The expression plasmid used here was pECα’, in which the cDNA encoding the β-conglycinin α’ subunit was subcloned into the expression vector pET21d(+) (Novagen) between the NcoI and BamHI sites.¹¹ The expression plasmid pECα’ was used as the template for site-directed mutagenesis. First, the EPR sequence was changed to DPR, then DPI was changed to DPR, and finally the DYR to DPR substitution was completed. Site-directed mutagenesis with synthetic oligonucleotide primers was done using a QuickChange Site-Directed Mutagenesis kit (Stratagene). Replacement of nucleotides in primers was designed as follows; ¹⁴Glu → Asp: GAA → GAT; ³⁶⁰Ile → Arg: ATC → CGC; ¹⁸⁷Tyr → Pro: TAC → CCC. The mutated DNA sequences were confirmed a DNA sequencer (Applied Biosystems, Model 310).

The expression plasmid pECα’, or the mutated pECα’ encoding the DPR-introduced α’ subunit, was used to transform the E. coli host strain, HMS174(DE3), which was grown as 2.8L Luria-Bertani (pH 7.0) cultures in the presence of 50 µg/ml ampicillin at 37°C, and induced with 1 mM IPTG for 48 h at 20°C. The induced cells were harvested by centrifugation. Portions of the cells were boiled in SDS sample buffer (62.5 mM Tris base, 2% SDS, 10% glycerol, 0.2 M 2-mercaptoethanol), and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with 11% acrylamide gels. Both the wild-type and DPR-expressing α’ subunits were recognized as single bands by anti-recombinant α’ subunit serum (data not shown).

Recombinant wild-type and DPR-expressing α’ subunits were purified. Cells were resuspended in buffer A (35 mM sodium phosphate, pH 7.6, 0.2 M NaCl, 10 mM 2-mercaptoethanol, 1 mM EDTA, 0.1 mM p-amidinophenyl...
methanesulfonyl fluoride hydrochloride (APMSF), 3 μg/ml leupeptin and 0.02% NaN₃ in a volume of 7 ml per gram of cells (wet weight), and disrupted by sonication on ice. Insoluble material was separated from the soluble fraction by centrifugation, and dissolved in SDS sample buffer. Both the recombinant wild-type and modified proteins were mainly recovered in the soluble fraction (Fig. 2, lanes 2 and 6). The expressed proteins were initially fractioned using ammonium sulfate (15–45% saturation). Precipitated proteins were collected by centrifugation, and resuspended in buffer B (35 mM sodium phosphate, pH 7.6, 0.28 M NaCl, 10 mM 2-mercaptoethanol, 1 mM EDTA, 0.1 mM APMSF, 3 μg/ml leupeptin, and 0.02% NaN₃). The suspension was dialyzed overnight against buffer C (35 mM sodium phosphate, pH 7.6, 0.1 M NaCl, 10 mM 2-mercaptoethanol, 1 mM EDTA, 0.1 mM APMSF, 3 μg/ml leupeptin and 0.02% NaN₃). The dialysate was clarified by centrifugation, and the resulting protein solution was fractionated by anion-exchange high performance liquid chromatography (HPLC) on a HiLoad 26/10 Q Sepharose high performance column (Amersham Pharmacia Biotech) equilibrated with buffer C. The elution was done with a linear gradient of 0.1–0.6M NaCl over a period of 250 min. Fractions containing the recombinant DPR-expressing α’ subunit were identified by SDS-PAGE. The purity of both wild-type and modified proteins exceeded 90% (Fig. 2, lanes 4 and 8). Purified proteins were dialyzed against pure water and lyophilized. This resulted in 4.9 mg of the wild-type protein and 6.1 mg of the modified protein being obtained from 1 L of culture.

To determine whether DPR could be released effectively from the modified α’ subunit as was predicted, the subunit was digested with trypsin. The wild-type or modified proteins (5 mg/ml) were adjusted to pH 8.0 and digested with trypsin (E/S = 1/50 [W/W], TPCK treated, from bovine pancreas, Sigma) for 12 h at 37°C, with the reaction being stopped by boiling. To isolate the released DPR, the digest (0.4 ml) of the modified α’ subunit was fractionated by reverse-phase HPLC on an octadecyl silica (ODS) column (Cosmosil SC18-AR-II, 4.6 x 150 mm, Nacalai Tesque). The column was eluted with a linear gradient of acetonitrile (1%/min), containing 0.1% TFA at a flow rate of 1 ml/min. The fraction eluted at the same retention time as synthetic DPR was collected (Fig. 3). It was confirmed using a 492 protein

---

**Fig. 1.** Three Sequences Similar to DPR and Putative Secondary Structures in a Soybean β-Conglycinin α’ Subunit.

“β” and “H” show β-structure and α-helix, respectively.

**Fig. 2.** SDS-PAGE Analysis of the Wild-type and the DPR-Expressing β-Conglycinin α’ Subunit.

Recombinant wild-type (lanes 1–4) and modified (lanes 5–8) subunits were expressed in E. coli HMS174 (DE3). Lane M, protein marker; lanes 1 and 5, total cell proteins after expression; lanes 2 and 6, soluble cell proteins; lanes 3 and 7, insoluble cell proteins obtained by sonicating the cells; lanes 4 and 8, wild-type and modified α’ subunits purified by Q Sepharose column chromatography.

---

<table>
<thead>
<tr>
<th>Wild-type</th>
<th>DPR-introduced</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>S</td>
</tr>
<tr>
<td>175</td>
<td>83</td>
</tr>
<tr>
<td>83</td>
<td>47.5</td>
</tr>
<tr>
<td>47.5</td>
<td>32.5</td>
</tr>
<tr>
<td>32.5</td>
<td>16.5</td>
</tr>
<tr>
<td>16.5</td>
<td></td>
</tr>
</tbody>
</table>

---
sequencer (Applied Biosystems) that the fraction was pure DPR. The overall yield of purified DPR was approximately 39%. Namely, 1.2 mole of DPR was obtained per one mole of the modified /C11 subunit. On the other hand, DPR was not detected in the tryptic digest of the wild-type recombinant /C11 subunit (data not shown). Thus, it is expected that DPR would be released in the intestine after ingestion of modified /C11 subunits.

In a previous study, we introduced the VPDPR sequence into the soybean proglycinin A1aB1b subunit by site-directed mutagenesis to potentiate hypocholesterolemic activity.8) However, after expression in E. coli, this modified proglycinin A1aB1b subunit was recovered in the insoluble fraction. Here, the DPR-expressing /C11 subunit was recovered in the soluble fraction, suggesting that it was folded normally. The site of the EPR sequence is situated at the end of the extension region, which is suggested to have a minor role in proper folding.11) According to the crystal structure of the /C12-conglycinin/C11 subunit, it is predicted that the DYR sequence is situated at the AB loop in the N-terminal /C12-barrel and that the DPI sequence is in the loop between the N-domain and C-domain.12) These observations suggest that no significant change occurred in the overall conformation of the /C11 subunit when the DPR sequences were introduced.

In a previous study, 0.4 mole of VPDPR was recovered from one mole of the modified glycinin A13B1b subunit containing one VPDPR sequence, after digestion with trypsin and chymotrypsin.5) The yield of DPR from the tryptic digest of the wild-type recombinant /C11 subunit containing three DPR sequences after trypsin digestion was 1.2 mole. DPR and VPDPR have hypocholesterolemic effects at a dose of 50 mg/kg and 100 mg/kg, respectively.5) Taken together, these results suggest that the DPR-expressing /C12-conglycinin /C11 subunit is superior to the VPDPR-expressing glycinin A13B1b subunit with respect to hypocholesterolemic activity. In this study, we introduced 3 DPR sequences by single amino acid replacement. We could introduce five additional DPR sequences into homologous sites by replacing single amino acid residues at the 12nd, 37th, 43rd, 81st, and 84th position in IPR, QPR, FPR, DER, and HPR sequences, respectively, in the extension region of the /C11 subunit. However, these sites are not preceded by protease-sensitive residues. Therefore, another substitution of the preceding residues would be required to facilitate the release of DPR from these sites by proteases.

On the basis of the minimum effective dose of DPR required to mediate a hypocholesterolemic effect in mice, the amount of DPR released from the modified /C11 subunit might not be sufficient for biological activity. Recently, we found that DPR stimulated the secretion of bile acids (unpublished observations). The /C12-conglycinin /C11 subunit was reported to activate LDL receptors in a human hepatoma cell line (Hep G2).13) Furthermore, /C12-conglycinin was found to have hypocholesterolemic activity in rats.14) It will be interesting to find to what extent the hypocholesterolemic activity of /C12-conglycinin in vivo is potentiated by this genetic modification. This will need to be tested after production of modified /C12-conglycinin in plants, since the yield in E.coli is too small for an in vivo study.

Acknowledgment

This work was supported in part by grants from the Ministry of Education, Science, and Culture of Japan to M.Y. and Y.T., and a PROBRAIN grant from the Bio-oriented Technology Research Advancement Institution to M.Y.

References

2) Erlanson-Albertsson, C., and Larsson, A., The activation