Recombinant curculin heterodimer exhibits taste-modifying and sweet-tasting activities

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Abstract **Curculin from *Curculigo latifolia* is a unique sweet protein that exhibits both sweet-tasting and taste-modifying activities. We isolated a gene that encodes a novel protein highly homologous to curculin. Using cDNAs of the previously known curculin (designated as curculin1) and the novel curculin isoform (curculin2), we produced a panel of homodimeric and heterodimeric recombinant curculins by *Escherichia coli* expression systems. It was revealed that sweet-tasting and taste-modifying activities were exhibited solely by the heterodimer of curculin1 and curculin2.**

Keywords: Curculin; Taste-modifying protein; Sweet taste; Heterodimer; Recombinant protein; Reconstitution

1. Introduction

So far, seven sweet proteins have been discovered in the fruits of tropical plants. Among them, miraculin is a taste-modifier but does not elicit sweet taste per se, whereas thaumatin, monellin, mabinlin, pentadin, and brazzein are sweet-tasting proteins without taste-modifying activity (see for review [1]). Curculin from *Curculigo latifolia*, which grows in a limited area of Western Malaysia, is a unique sweet protein because it exhibits both sweet-tasting and taste-modifying activities [2]. Structural basis of sweet-tasting activity has been extensively studied for thaumatin [3,4], monellin [5–9], and brazzein [10], and residues important for sweet taste have been reported previously [11–15]. In contrast, little or no structural and mutational study has so far been performed for the taste-modifying proteins, and therefore the taste-modifying mechanisms are as yet unknown.

Protein chemical data have revealed that curculin is a dimer of a polypeptide with 114 residues [2]. Although cDNA clones for curculin have been isolated and sequenced [16], expression of this protein in recombinant hosts has never been reported yet.

In the present study, we attempt to produce recombinant curculin in an *Escherichia coli* expression system. We found a novel protein that was contained in the native curculin fraction and isolated a gene that encodes this protein. It was revealed that the amino acid sequence of this protein is highly homologous to the previously reported amino acid sequence of curculin. Hereafter, the previously reported and newly described curculin isoforms will be designated as curculin1 and curculin2, respectively. Using cDNAs of these proteins, we produced a panel of homodimeric and heterodimeric recombinant curculins and examined their sweet-tasting and taste-modifying activities.

2. Materials and methods

2.1. Preparation of native curculin and curculin1 cDNA

Native curculin was isolated from the pulp of *Curculigo latifolia* by the method reported previously [2]. Curculin1 cDNA was chemically synthesized based on the nucleotide sequence of the curculin1 gene [16].

2.2. cDNA cloning and sequencing of curculin2

Total RNA was extracted by the phenol–SDS method [17] from the pulp of *Curculigo latifolia*. Poly(A)$^+$RNA was purified by Oligotex-dT30 Super purchased from Takara. Double-stranded cDNA was synthesized using a cDNA synthesis kit (Takara). N-terminal amino acid sequencing suggested that the native curculin fraction contains a novel protein that is highly homologous to curculin with amino acid substitutions at seven positions within the N-terminal 30 amino acid residues. Based on the amino acid sequence GQTLYAG, which contained two (underscored) of the seven substituted amino acid residues, oligodeoxynucleotide primers, 5′-GGICARACICTITAYGCIGG-3′ (where R = A or G, Y = C or T and I = inosine), were designed. These oligodeoxynucleotide primers along with those designed on the basis of the nucleotide sequence of the curculin1 gene [16] were used for isolation of the gene encoding the putative curculin isoform (curculin2). The curculin2 cDNA fragment was amplified by polymerase chain

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Abbreviations: CD, circular dichroism; DEAE, diethylaminoethyl; GSH, glutathione reduced form; GSSG, glutathione oxidized form; HPLC, high-performance liquid chromatography; MALDI, matrix assisted laser desorption ionization; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; TOF-MS, time-of-flight mass spectrometry; UV, ultraviolet

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reaction (PCR) and then inserted into the vector, pMOSBlue purchased from Amersham Biosciences, and sequenced by a *Bca*BEST dideoxy sequencing kit (Takara). The nucleotide sequence for the novel curculin gene has been submitted to the GenBank under Accession No. AB181490.

2.3. Protein expression and purification

BL21(DE3) *E. coli* strain was used for expression of the recombinant curculin1 and curculin2, in which expressed curculins formed inclusion bodies. After solubilization of them by 6 M guanidinium chloride, two kinds of curculin homodimers were reconstituted by dilution with refolding buffers (50 mM Tris–HCl, pH 8.0, 3 mM glutathione reduced form, GSH and 0.3 mM glutathione oxidized form, GSSG containing 2 M guanidinium chloride for curculin1 homodimer and 60% ethylene glycol/1 M LiCl for curculin2 homodimer, respectively) at 16 °C. For reconstitution of curculin heterodimer, denatured curculin1 and curculin2 were mixed prior to refolding and then the mixture was diluted with refolding buffer (50 mM Tris–HCl, pH 8.0, 3 mM GSH and 0.3 mM GSSG containing 2 M guanidinium chloride). Reconstituted curculin1 homodimer and heterodimer were subjected to cation-exchange chromatography using a Mono S column (Amersham Biosciences) for purification. Purification of curculin2 homodimer was performed with anion-exchange chromatography using diethylaminoethyl (DEAE) column (Shimadzu). The purified curculin dimers were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing and non-reducing conditions.

2.4. Measurements of sweet-tasting and taste-modifying activities

The sweet-tasting and taste-modifying activities of curculin dimers were assayed using four subjects as described previously [18]. Prior to evaluation, subjects tasted repeatedly a series of standard sucrose solutions (0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, and 0.50 M), and remembered their sweetness intensity. Then, 5 ml of 4 μM each curculin dimer dissolved in 50 mM phosphate buffer of pH 6.0 was sipped, held in the mouth for 3 min and spat out. At 15 s after sipping the solution, subjects evaluated sweetness of curculin solution itself by choosing a standard solution with an equivalent intensity of sweetness. After curculin solution was spat out, subjects rinsed their mouth with water, sipped 5 ml of milli-Q water or 50 mM citric acid and evaluated sweetness intensity as described above for measurement of taste-modifying activities. At least 1 h was interposed between each test.

2.5. Measurements of CD spectra of curculin dimers

Curculin dimers were dissolved in 50 mM phosphate buffer of pH 6.0 containing 0.1 M NaCl. Measurements of ultraviolet (UV) circular dichroism (CD) spectra of curculin dimers were performed in a 1 mm quartz cuvette at 20 °C using spectropolarimeter (J-725, JASCO). After subtraction of the spectrum of the buffer alone, data were represented as mean residue ellipticities.

2.6. Identification of disulfide linkage between curculin subunits

Curculin dimers were subjected to proteolysis by pepsin at pH 2.0. Proteolytic fragments were separated by reverse phase high-performance liquid chromatography (HPLC) using Vydac C4 column. Disulfide linkages between curculin subunits were identified by amino acid sequencing and molecular weights measurements of the fragments containing cystine residues using peptide sequencer (Model 491 Procise cLC, Applied Biosystems) and matrix assisted laser desorption ionization-time-of-flight mass spectrometry (AXIMA-CFR-NC, Shimadzu), respectively.

3. Results and discussion

Amino acid sequence analysis revealed that there existed two kinds of amino acid sequences in native curculin from the pulp of *Curculigo latifolia*. One is consistent with the amino acid sequence reported previously [2] and the other contains amino acid substitutions at seven positions within the N-terminal 30 amino acid residues. Using the oligodeoxynucleotide primers derived from the unique amino acid sequence (GQTLYAG) close to the N-terminus, a gene encoding the novel protein was cloned and sequenced. The deduced amino acid sequence of the novel gene has 77% identity to that of curculin reported previously (Fig. 1). In contrast to the previously reported curculin isoform, this protein is acidic with a calculated isoelectric point of 4.7. The basic and acidic curculin polypeptides were designated as curculin1 and curculin2, respectively. Curculin has so far been reported to be a disulfide-linked dimer [2]. Since all of the four cysteine residues at positions 29, 52, 77 and 109 are conserved between curculin1 and curculin2, it is possible that curculin2 is capable of forming a homodimer as well as a heterodimer together with curculin1. To identify sweet-modifying substance, we attempted to produce curculin dimers as recombinant proteins.

Recombinant curculin1 and curculin2 were separately expressed in *E. coli* as inclusion bodies. By a guanidine-denaturation and oxidative refolding procedures, we succeeded in preparation of the three possible dimeric isoforms of curculin1 and curculin2, i.e., the homodimers of curcurin1 (curculin1-1) and of curculin2 (curculin2-2) along with the heterodimer consisting of these subunits (curculin1-2) (Fig. 2). Inspection of the peptide sequencing and mass spectrometric data of the proteolytic fragments of the recombinant curculin dimers indicated that Cys29 and Cys52 forms an intra-chain disulfide bridge, while Cys77 and Cys109 in one subunit are linked to Cys109 and Cys77 in the other subunit, respectively, forming inter-chain disulfide bridges (data not shown). These three disulfide-linked dimers exhibited similar far UV CD spectra, indicating that they assume similar folds (Fig. 3).

Sweet-tasting and taste-modifying activities of three kinds of curculin dimers were estimated according to the method previously reported [18]. The results are summarized in Fig. 4. It was found that the curculin1-2 heterodimer has both sweet-tasting and taste-modifying activities, while the homodimeric forms, i.e., curculin1-1 and curculin2-2, had neither of those activities. The native curculin protein has been reported to be 35 000 and 430 times sweeter than sucrose on molar and weight bases [18]. Sweet-tasting and taste-modifying activities of the recombinant curculin1-2 heterodimer are comparable with those of native curculin.

In the present study, we have shown that the heterodimer is the only active form of curculin. Although curculin2 possesses one possible N-glycosylation site at position81, glycosylation is

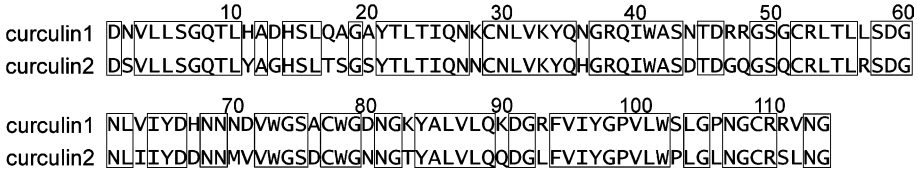

Fig. 1. Amino acid sequences of curculin1 and curculin2. Conserved amino acid residues between two sequences are boxed.

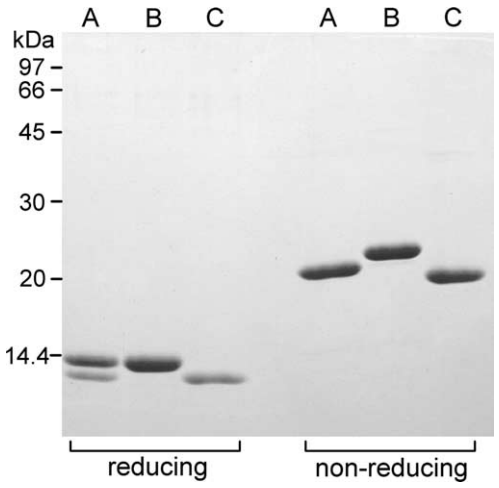

Fig. 2. Non-reducing and reducing SDS–PAGE of curculin dimers. A, B, and C represent curculin1-2 heterodimer, curculin1 homodimer and curculin2 homodimer, respectively. Positions of molecular weight markers are shown at the left side.

not essential to the sweet-tasting nor taste-modifying activity if any. Since curculin1 and curculin2 are abundant in basic and acidic residues, respectively, molecular surface properties are significantly different among the three curculin isoforms. Especially, curculin1-2, the active isoform, exhibits a bipolar charge distribution, which might be important for the interaction with a putative sweet taste receptor.

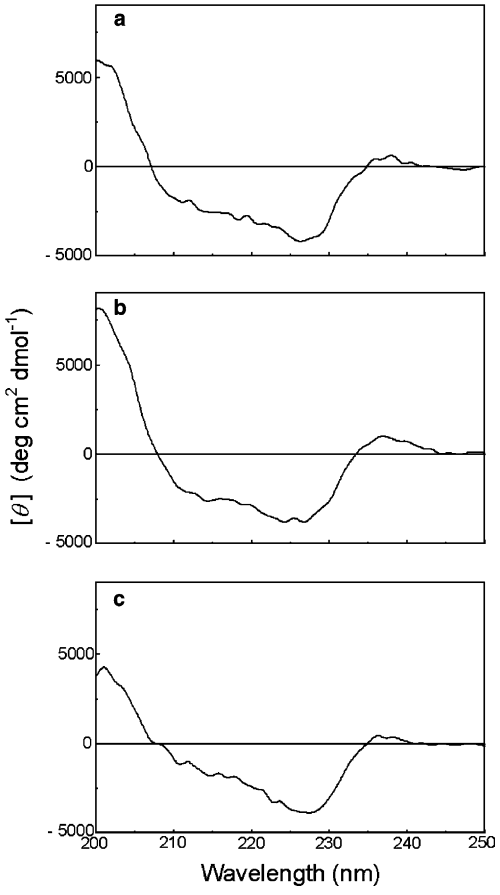

Fig. 3. Far UV CD spectra of curculin dimers: (a) curculin1-2, (b) curculin1-1, (c) curculin2-2. Protein concentration of each sample was 0.1 mg/ml.

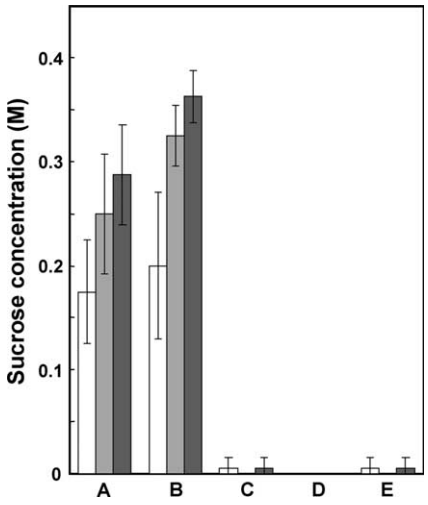

Fig. 4. Evaluation of sweet-tasting and taste-modifying activities of curculin dimers. Mean sweetness intensity is represented as concentration of standard sucrose solution with ±S.D. bar ($n = 4$). Open bar: sweetness of curculin dimer itself, gray bar: sweetness induced by water, dark gray bar: sweetness induced by 50 mM citric acid. (A) curculin1-2, (B) curculin1-2 + 100 mM NaCl, (C) curculin1-1, (D) curculin1-1 + 100 mM NaCl, (E) curculin2-2 + 100 mM NaCl. Because of low solubility, sweet-tasting and taste-modifying activities of curculin2-2 were evaluated with NaCl.

In conclusion, the establishment of production of recombinant curculin will open up a new way in a field of research of structure–function relationships of sweet-tasting and taste-modifying proteins and in industrial applications of curculin as a low-calorie sweetener.

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