4. Solutions on Curculin Modification **

Summary

Rank	Solution	Solution proposed by	Non caloric or very low calorie	Stable at low pH (~pH 3.0)	Sensory profile closer to sugar	No licorice taste or Aftertaste	Thermal stability	Stable to UV exposure	Low cost	Robust supply chain	Solubility
22	Production of Curculin	Yokohama National University		**More sweet at acidic condition**	20,000 times sweeter than sucrose	**Sweet taste of curculin disappeared a few minutes after holding it in the mouth; Modifying sour taste**					
39	Mass production of Curculin	**Adeka Corp**	Yes							**Mass production**	

Curculin (1/2)

Introduction

- Substances of high molecular weight do not stimulate taste cells and hence have no taste. This is true for most proteins, although a limited number of proteins stimulate taste cells.
- **One type is a protein which elicits a sweet taste. Four sweet proteins have been discovered so far: thaumatin, monellin, mabinlin, and pentadin**.
- Another type stimulates taste cells in taste-modifying fashion; only one of these, called miraculin, had been found. Miraculin has the unusual property of modifying a sour taste into a sweet taste.
- In previous studies, we have completely purified miraculin and determined its amino acid sequence. **Miraculin itself has no sweet taste, and the sweet proteins described above have no taste-modifying activity.** Hence, it has been suggested that the two types of proteins are not related to each other.
- **In this study, we have found a new type of protein which elicits a sweet taste and also has taste-modifying activities.**
- **This protein has been isolated from the fruits of Curculigo latifolia.** This plant is a stemless herb which grows wild in Western Malaysia and bears fruits weighing -1 g.
- **Native people eat the fruits to give a sweet taste to sour foods. The pulp of the fruit tastes sweet.**
- In addition, after chewing the pulp, water elicits a sweet taste, and black tea tastes sweet without sugar. Sour substances such as citric acid or ascorbic acid induce a stronger sense of sweetness. **In spite of these interesting properties, no attempt to purify the active principle has been reported.**
- **In this study, we have succeeded in purifying the active principle and name it curculin. We also have determined the complete amino acid sequence of curculin**. Curculin is a dimer of a polypeptide with 114 residues.
- Purified curculin is practically insoluble in deionized water and soluble in the salt solution. The sequence analysis indicates that it is composed of a single polypeptide.
- The taste-modifying action of curculin is explained, although this mechanism is highly speculative. Like miraculin, curculin is assumed to have two binding sites: one site binds to a receptor site of the sweet receptor protein, and **another site binds to a site near the sweet receptor site. The latter binding is rather strong; and hence, curculin once applied to the tongue is not easily detached from the receptor membranes.**

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Curculin (2/2)

Introduction

- **A new type of protein which elicits a sweet taste and also has taste-modifying activity have been found.**
- **This protein has been isolated from the fruits of Curculigo latifolia.** This plant is a stemless herb which grows wild in Western Malaysia and bears fruits weighing -1 g.
- **Native people eat the fruits to give a sweet taste to sour foods.**
- In addition, after chewing the pulp, water elicits a sweet taste, and black tea tastes sweet without sugar.
- **Sour substances such as citric acid or ascorbic acid induce a stronger sense of sweetness.**
- **In spite of these interesting properties, no attempt to purify the active principle has been reported.**
- **Researchers have succeeded in purifying the active principle and name it curculin.**
- **Researchers also have determined the complete amino acid sequence of curculin**.
- **Curculin is a dimer of a polypeptide with 114 residues.**
- Purified curculin is practically insoluble in deionized water and soluble in the salt solution.
- The sequence analysis indicates that it is composed of a single polypeptide.
- The taste-modifying action of curculin is explained, although this mechanism is highly speculative. Like miraculin, curculin is assumed to have two binding sites: one site binds to a receptor site of the sweet receptor protein, and **another site binds to a site near the sweet receptor site.**
- **The latter binding is rather strong; and hence, curculin once applied to the tongue is not easily detached from the receptor membranes.**

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4.1 Production of Curculin **

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Production of Curculin (2/4)

The solution to the problem is invented by Yokohama National University

Application in food & beverages.

Solution

Non-patent Published in 1990

Purification of Curculin:

- **Pulp was extracted with 0.5 M NaCl. The extract was colorless and tasted sweet. After ammonium sulfate fractionation, the sample was applied to a CM-Sepharose column.** The bound substances were eluted from the column as one sharp peak (peak B) by a linear gradient of NaCl. The fractions in peak B tasted sweet. The fractions under this peak were collected, and ammonium sulfate was added to -80% saturation.
- **The precipitate formed was dissolved in 10 mM phosphate buffer, and the solution was subjected to gel filtration using a Sephadex G-100 column.**
- The peak fractions were collected and used as a purified curculin sample. Five milligrams of purified curculin were obtained from 30 g of pulp, wet weight.
- **The purity of purified curculin was checked in various ways. S-Carboxamidomethylated curculin was applied to reverse phase HPLC.**
- The purity was also checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of 8 M urea.
- Analysis of the NHz-terminal amino acid sequence of the purified protein in a gas-phase sequenator revealed only one amino acid sequence.

Curculin was easily extracted with 0.5 M NaCl after the pulp was washed with water. This washing of the pulp with water and subsequent extraction with NaCl greatly increased the -fold purification of curculin.

Amino Acid Sequence of Curculin:

- *The sequence of the first 20 residues was determined for S-carboxamidomethylated curculin. Aspartic acid is the amino-terminal amino acid. Modified curculin was digested with lysyl endopeptidase, LYchymotrypsin, and trypsin. Peptides produced were well-separated on HPLC - lysyl endopeptidase, cY-chymotrypsin, and trypsin.*
- *The sequences were confirmed by determining the amino acid compositions of the peptides. Analyses of the amino acid compositions of all peaks obtained by digestion with trypsin were carried out; and on the basis of the results, peptide T-I was used to obtain the sequences of peptides LEP-3 and CH-7.*

Properties of Curculin:

- *The molecular weight of native curculin was determined to be 27,800 by low-angle laser light scattering, whereas that estimated by SDS-PAGE was approx. 12,000. Hence, native curculin is a dimer of a 12000 Da polypeptide.*
- *The amino acid composition of curculin was determined on a 6 N HCl hydrolysate. Curculin contains a relatively high content of aspartic acid, leucine, and glycine. Carbohydrates were not detected, indicating that curculin is not a glycoprotein.*

Source

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Production of Curculin (3/4)

Solution

Activities of Curculin:

- Purified curculin tastes sweet. **The sweetness of 10 PM curculin was equivalent to that of 0.2 M sucrose. Hence, curculin is 20,000 times sweeter than sucrose on a molar basis and 550 times sweeter than sucrose on a weight basis.**
- **The sweet taste of curculin disappeared a few minutes after holding it in the mouth. Then, application of water to the mouth elicted a sweet taste. The action of making water sweet lasted for - 5 min.**
- **Curculin also has the property of modifying a sour taste.**
- After **curculin was held in the mouth for 3 min and its sweet taste had disappeared, the sweet taste induced by acids was evaluated**.
- **The sweetness induced by 0.02 M citric acid after 10 pM curculin was held in the mouth was equivalent to that of 0.35 M sucrose. This taste-modifying action of curculin lasted for - 10 min.**
- **As described above, the sweetness of curculin disappeared a few minutes after holding it in mouth, but was recovered when water was applied to the tongue. This suggests that some substances in saliva suppress the sweet taste of curculin and that elimination of the substances recovers the sweetness.**
- NaCl solution (0.5 M) recovered the sweetness of curculin similar to water. On the other hand, 1 mM CaCl, or MgC & did not recover the sweetness of curculin. It is known that saliva contains 1 mM Ca'+; hence, Ca2+ and/or Mg2+ in saliva seems to suppress the sweetness of curculin.

Result

- **The active site of curculin weakly stimulates the sweet receptor site on the receptor membrane; and hence, only mild sweetness is induced.**
- **The presence of Ca2+ and/or Mg2+ in saliva suppresses the stimulation of curculin at the sweet receptor site, which results in the disappearance of the sweetness of curculin.** Application of water to the tongue leads to removal of the divalent cations in saliva from the tongue surface, and hence, the sweet taste of curculin is recovered.
- **Application of acid leads to changes in the conformation of the taste receptor membranes, which results in an increase in affinity of the active site of curculin for the sweet receptor site. Hence, acid induces a stronger sense of sweetness.**
- Elimination of acid from the tongue surface leads to detachment of the active site of curculin from the sweet receptor site, although curculin itself is not detached from the receptor membrane.

Source

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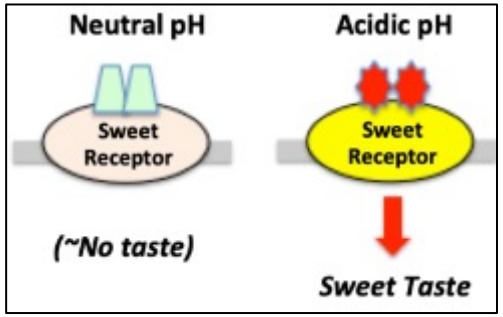
Production of Curculin (4/4)

Applications

- The sweetness of curculin disappeared a few minutes after holding it in mouth, but was recovered when water was applied to the tongue. This suggests that some substances in saliva suppress the sweet taste of curculin and that elimination of the substances recovers the sweetness.
- **A conformational change of curculin by acid may also contribute to the increase in affinity.**



Conventional Solutions

No protein with both sweet-tasting and taste-modifying activities has ever been found.

Advantages

Curculin itself elicits a sweet taste. After curculin, water elicits a sweet taste, and sour substances induce a stronger sense of sweetness.

Comment

- **A new taste-modifying protein named curculin was extracted with 0.5 M NaCl from the fruits of Curculigo latifolia and purified by ammonium sulfate fractionation, CM-Sepharose ion-exchange chromatography, and gel filtration.**
- The complete amino acid sequence of curculin was determined by automatic Edman degradation. It consists of 114 residues. **Curculin itself elicits a sweet taste. After curculin, water elicits a sweet taste, and sour substances induce a stronger sense of sweetness. No protein with both sweet-tasting and taste-modifying activities has ever been found.**

Source

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4.2 Mass production of Curculin **

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Mass production of Curculin

The solution to the problem is invented by Adeka Corp

Patent Published in 1996

Application in food & beverages.

Solution

- **The reference discloses about a process for producing a DNA molecule comprising the base sequence encoding curculin B, comprising separating a mRNA fraction containing curculin B mRNA from Curculigo latifolia, preparing single-stranded DNA from said mRNA using reverse transcriptase.** Preparing double-stranded DNA from said single-stranded DNA, Inserting said double-stranded DNA into a vector. Transforming a host with said vector having the double-stranded DNA inserted therein to produce a cDNA library. **Isolating from said library a cDNA having the base sequence of SEQ ID No. 4 or SEQ ID No. 5, using one or more synthesized probes containing a base sequence coding for a partial amino acid sequence elucidated from curculin A purified from Curculigo latifolia. Culturing a transformed cell or microorganism containing a recombinant DNA containing the base sequence of SEQ ID No. 4 or SEQ ID No. 5 whereby curculin B is produced by said transformed cell or microorganism.** Isolating curculin B from said transformed cell or microorganism.

- **The probe consists of an oligonucleotide having a first segment which consists of the sequence of SEQ ID NO:6 and a second segment which consists of the sequence of either SEQ ID NO:7 or SEQ ID NO:8.**

Tests

Western Analysis:

- **The cells obtained were suspended in 100 .mu.l of a 10 mM tris-1 mM-EDTA buffer solution (pH 7.5) containing 1 mM-PMSF (phenylmethanesulfonylfluoride) and destroyed by ultrasonication.**
- 3 .mu.l of the resulting liquid was separated by SDS-polyacrylamide gel electrophoresis.

Results

Western Analysis:

- **The results are shown by lane 1 and lane 2 in FIG. Lane 1 is a transfer of lane 3, while lane 2 is a transfer of lane 4 (for control). Lane 2 does not include any component reacting with the anti-curculin A antibody, while lane 1, as shown by the arrow mark to the left side, includes the component which reacts with the anti-curculin A antibody at a position corresponding to the protein appearing at the position of about 17 kd of lane 3. The results are shown in lane 3 and Lane 4 of FIG is a control lane and was obtained by destroying the E. coli YA21 strain transformed by a plasmid without the recombination procedure and separating the resulting liquid by SDS-polyacrylamide gel electrophoresis in the same manner as above.** As shown by the arrow mark at the left of lane 3, **a protein component is found at the position of about 17 kd.**

Source

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