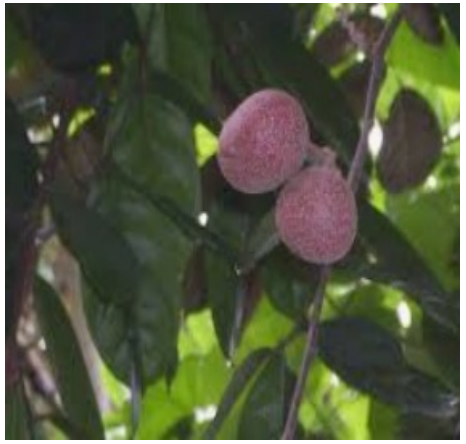
## 6. Solutions on mabinlin **

| 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 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 37 | Mass production of mabinlin | **Alkion BioInnovations** | | | 400 to 600 times sweeter than sucrose | | | | **Low cost** | **Mass production** | |
| 41 | Heat-stable mabinlin | Yokohama National University, Yokohama City University School of Medicine, Kunming Institute of Botany | | | Sweetness | | **Sweetness of mabinlins is unchanged at 48 hours incubation at 80°C** | | | | |

# Mabinlin

## Introduction

- Mabinlin is found in mature seeds of Capparis masaikai Ltvl. (local name mabinlang) which grows in a subtropical region of the Yunnan province of China.
- In a previous study, **Heat-stable homologue (mabinlin 11) was purified and showed that boiling mabinlin I1 did not abolish its sweet activity**.
- The complete amino acid sequence and disulfide array of mabinlin I1 was determined.
- In the present study, we have purified other homologues, mabinlin 1-1, 111 and IV.
- The sweet taste of mabinlin 1-1 was completely abolished by a l-h **incubation at 80°C while the taste of mabinlin I11 and mabinlin IV was unchanged by this treatment as was observed previously for mabinlin 1-1**.
- The amino acid sequences were determined of these homologues and the positions of the disulfide bridges.
- It was concluded that **replacement of a B-chain glutamine residue at position 47 by arginine contributes to the heat stability of mabinlin homologues**.



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## 6.1 Mass production of mabinlin **

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Solution

# Mass production of mabinlin (1/2)

**The solution to the problem is invented by Alkion BioInnovations**

*Published after 2017*

**Application in food & beverages.**

- **Alkion BioInnovations is a young biotech private company formerly known as Alkion BioPharma, founded in 2011 and founded in 2017 after being acquired by Evonik Industries AG.**
- The company is based in France.
- **The company produces environmentally friendly, GM-free bio-natural ingredients and seeks to revolutionized the nutrition market through its production of sweet proteins,** superfoods, flavors and other natural additives.
- They are developing new food and medical ingredients or API. In particular the company is working with a new natural sweet proteins : mabinlin from Capparis Masaikai, and is developing a new large scale method to produce medical cannabinoids.
- **Alkion has been selected as Beneficiary for the NeGenSweet (Laureate of i-Nov Program - Oct 2018). It offers a €718k grant+R&D loan. The project is the production of mabinlin extracts from plants produced in large scale bioreactors – Nov 2019.**
- Alkinnov (Alkion BioInnovations) develops and produces innovative ingredients (the new generation of natural intense sweeteners, plant-based proteins and natural additives) through a disruptive GMO-free process of plant biostimulation in bioreactors to revolutionize the nutrition market.
- Sarah Meryll Buet is CEO of Alkion BioInnovations**.**
- **Fruit from the Capparis Masaikai plant, contains the sweet protein mabinlin. Manbinlin is 400 to 600 times sweeter than sucrose.**
- **Alkion BioInnovations produces sweet-tasting proteins with biostimulation process, high-value flavours and plant proteins in a cost-effective way that can be labelled as organic.**
- The company develops and produces three types of ingredients for food manufacturers: natural intense sweeteners, flavours and plant-based proteins with a production process that involves **hydroponics and 'robotised' (or connected) bioreactors**. After obtaining stable callus, the company cultivated specific plant organs – often the leaf to be able to produce a very high quantity of compounds of interest with genes biostimulation. Finally, purified them if needed.
- **It's a patented process of in vitro plant tissue cultures in bioreactors. The company is focusing its technology on high-value ingredients that have scale-up issues. The process differs to the methods being used by suppliers to scale up high-value, low-quantity molecules.** Some of Alkion BioInnovations most advanced products in its portfolio are also sweeteners.

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# Mass production of mabinlin (1/2)

## Solutions

- **Alkinnov's new bioreactor "Alkaburst 2.0" has been designed and patented to produce high volumes production where cost can be divided by a factor of 50 to first target unsuppliable natural expensive sweeteners & flavors.**
- Indeed the performance of production yields can reach up to 1000 times the ones on soil today with room for improvement using A.I. and biostimulation.
- The project focuses on the development of a 100t/year pilot plant for 5 major food addition/flavors to reshape the consumption of the natural compounds in food. In addition, the technology has been validated for 70 other leaves, shoots & root varieties and has the potential to support in-vitro cultivation of any plant tissue, covering numerous applications in pharmaceuticals, fragrances, pest-control, phyto-protection and food additives, among others.
- Stevia Rebaudiana receives a strong demand despite its licorice after-taste and its potential effect on microbiome which slowed down its commercial progression. An alternative to Stevia is more and more demanded. **To offer these alternative industrial natural solutions, Alkion has decided to work on a non-bitter Stevia but also has selected three exotic plants which contain powerful harmless natural sweet proteins that can not be industrially exploited due to extremely low production yield and difficult access to the plant.**
- Its R&D team, supported by a European scientific community, is developing a non-GMO protocol and an large-scale industrial production device to produce and supply these natural food sweeteners. Its patented technologies achieve an exceptional production yield compared to classical solutions, making accessible the plant super-capacities when they are trained and developed in the best culture conditions without genetic modification.
- The team expects to launch with international partners new natural affordable sweeteners to support the European objectives of diabetes & obesity decrease.
- The potential impacts will be the validation of 3 to 4 new natural sweeteners to offer and sell them to the food & beverages industry. It could have an important impact on the sugar reduction in beverages and therefore on obesity and diabetes.

➢ The company produces sweet-tasting proteins with biostimulation process, high-value flavours and plant proteins in a cost-effective way that can be labelled as organic.

Source1 Source2

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## 6.2 Heat-stable mabinlin **

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# Heat-stable mabinlin (1/3)

**The solution to the problem is invented by Yokohama National University, Yokohama City University School of Medicine, Kunming Institute of Botany**

**Solution**

**Application in food & beverages.**

*Non-patent Published in 1994*

**Purification of mabinlin homologues:**

- The elution profile from carboxymethyl- Sepharose CL-6B (Pharmacia) showed four main peaks. Fractions of peak 2 were further purified. The fractions were collected and ammonium sulfate was added to 70% saturation. The precipitate was collected by centrifugation at 22000Xg for 90min and dissolved in 10 mM KH,POJNa2HP0, (pH 6.8). The solution was applied to a Bio Gel P-30 column and two peaks were eluted.
- The fractions from the first peak, named mabinlin 1-1, were collected and desalted by ultrafiltration using an Amicon YM-5 filter. The solution was lyophilized as pure mabinlin 1-1. The yield of pure mabinlin 1-1 was 5.4 mg from 0.4 g seeds. The fractions of peaks 3 and 4 were also collected and desalted by ultrafiltration. The solutions were lyophilized as pure mabinlin I11 and mabinlin IV. The yield of pure mabinlin 111 and mabinlin IV was 5.0 mg and 2.2 mg from 0.4 g seeds, respectively.

**Reverse-phase HPLC:**

- Mabinlin 1-1, 111 or IV was subjected to reverse-phase HPLC on a TSK gel TMS-250 column (4.6 mmX75 mm). The protein was eluted from the column by a linear gradient of CH,CN (10-50%) containing 0.05% CF,CO,H for 25 min at a flow rate of 1 mumin. Protein was monitored by measuring the absorbance at 210 nm. Reduced and S-pyridylethylated mabinlin 1-1, I11 or IV were separated on a TSK gel TMS-250 column (4.6 nmxlOO mm). The peptides in hydrolysates after enzymic digestion were separated on a Vydac 218TP54 column (4.6 nmX250 mm) and purified again on the same column. The peptides were eluted from the column by a linear gradient of CH,CN containing 0.05% CF,CO,H at a flow rate of 1 ml/min. The eluted peptides were monitored by measuring the absorbance at 210 nm and 280 nm. Each peak was collected manually

**Isolation of cystine-containing peptides:**

- Trypsin digestion of mabinlin 1-1 was performed in 0.2 M ammonium acetate (pH 6.5) containing 10 mM CaC1, at 37°C for 24 h. The protein concentration was 1 mg/ml, and the enzyme-to-substrate ratio was 1 : 50 (by mass). Thermolysin digestion of peptide T-21 was performed in 0.2 M ammonium acetate (pH 6.5) containing 10 mM CaC1, at 37°C for 24 h. The peptide concentration was 40 nmol/ml, and the enzyme-to-substrate ratio was 1 : 20 (mol/mol). The digested samples were injected into a HPLC column to isolate the peptides.

Source

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# Heat-stable mabinlin (2/3)

## Tests

**Determination of amino acid sequence:**

- **Conversion of pyroglutamyl residues to glutumyl residue -** The A-chain was incubated in 1 M HCl/methanol (1 : 11, by vol.) at 35°C for 24 h , and the solution was lyophilized. **Enzymic cleavage -** Pyroglutamate aminopeptidase digestions of the B-chain and peptide L-1 were performed in 0.1 M sodium phosphate (pH 8.0) containing 5 mM dithiothreitol and 10 mM EDTA at 35°C for 24 h and at 37°C for 4.5 h, respectively. The peptide concentrations were 25 mM and 54 mM for B-chain and peptide L-1, respectively, and the enzyme-to-substrate ratio was 0.2 mU/nmol. a-Chymotrypsin digestion of Achain was performed in 25 mM ammonium bicarbonate (pH 7.8) at 25°C for 1 h. The peptide concentration was 1 mg/ml, and the enzyme-to-substrate ratio was 1 :200 (by mass). Trypsin digestion of A-chain was performed in 50 mM Tris/HCl (pH 8.0) containing 10 mM CaCI, at 37°C for 24 h. The peptide concentration was 1 mg/ml, and the enzyme-to-substrate ratio was 1 : 50 (by mass). Lysyl endopeptidase digestion of B-chain was performed in 50 mM Tris/ HCl (pH 9.0) containing 2 M urea and 2 mM EDTA at 35°C for 20 h. The peptide concentration was 1 mg/ml, and the enzyme-to-substrate ratio was 1 : 200 (mol/mol). Endoproteinase Glu-C digestion of B-chain was performed in 25 mM ammonium bicarbonate (pH 7.8) at 25°C for 3.5 h. The peptide concentration was 1 mg/ml, and the enzyme-to-substrate ratio was 1 : 25 (by mass). The digested sample was injected into a HPLC column to isolate the peptides.
- **Determination of the C-terminal amino acid sequence -** The C-terminal amino acid sequences of the A-chains and B-chains were determined by using carboxypeptidase W. The samples were dissolved in 0.1 M pyridine/acetic acid (pH 4.0). The concentration of the peptides was 250 nmol/ ml. Carboxypeptidase W was added in the enzyme-to-substrate ratio of 1 : 10 (by mass). The reaction mixture was incubated at 30°C. Aliquots were taken at 0, 10, 30, 60 and 120 min. The reaction was terminated by adding acetic acid to a final pH of pH 2.0, and the released amino acids were analyzed.
- **Measurement of sweet activity:** The sweetnesses of mabinlins 1-1, 11, I11 and IV were assayed. **The sweetness of mabinlin homologues was evaluated by comparing its sweetness with that of a series of standard sucrose solutions (0.05- 0.3 M). The heat stability of mabinlins 1-1, 11, I11 and IV was evaluated**. **Each mabinlin homologue (0.8 mM) was incubated at 80°C for various time periods. The sample solution was cooled to 20°C and the sweetness was evaluated**.
- **Measurement of CD spectra:** Mabinlins 1-1, 11, I11 and IV, with and without heating at **80°C for 1 h**, were prepared. Circular dichroism (CD) spectra were recorded at 20°C on a Jasco J720 spectropolarimeter interfaced with an NEC PC-9801 FX personal computer. Measurements were carried out in a cell with a 0.1-cm path length using 13- 15 pM mabinlin. Four scans were averaged for base-line correction. The results were expressed as molar ellipticities. The a-helix contents were estimated by fitting the experimental data to reference spectra.

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# Heat-stable mabinlin (3/3)

**Result**

**Purity of mabinlin homologues:**

- The purity of the purified mabinlins 1-1, 111 or IV was confirmed by various methods. The elution profiles of the homologues in reverse-phase HPLC indicated that the samples were very pure. Each sample also showed a single band on SDSPAGE.

**Heat stability of mabinlin homologues:**

- **The sweetness of mabinlin homologue at 0.8 mM was equivalent to that of 0.3 M sucrose**. There was no essential difference in the sweetness among mabinlin homologues. The sweetness of mabinlin 1-1 is completely abolished by incubation for 1 h at 80°C. The **sweetness of mabinlins 11-IV is unchanged by a 1-h incubation at 80°C**.

- **The sweetness of these homologues was unchanged even by a 48 h-incubation at 80°C**

**Changes in the a-helix contents of mabinlin homologues with an increase of temperature:**

- The spectrum is typical of an a-helical structure with a double minimum approximately at 222 nm and 208nm and a maximum at approximately 193nm. The amount of a-helix content was estimated to be 49%. Heating drastically changed the CD spectrum to a spectrum typical of a random coil with a minimum around approximately 198 nm. This implies that the a-helical structure of mabinlin 1-1 was completely destroyed by heating. The CD spectrum of mabinlin II is very similar to that of mabinlin 1-1. The content of a-helix of mabinlin II was 56%. The CD spectrum was unchanged by heating at 80°C for 1 h, indicating that the secondary structure of mabinlin I1 is unchanged by the heat treatment. Like mabinlin 11, the CD spectra of mabinlin III and mabinlin IV were unchanged by the heat treatment.

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