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Crystallization and preliminary X-ray analysis of the thermostable sweet protein mabinlin II

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Mabinlin II is a sweet protein with the highest known thermostablility and is isolated from the seeds of *Capparis masaikai* Levl. grown in south China. Two crystal forms of mabinlin II were obtained using the hanging-drop vapour-diffusion method. One of them diffracts to 2.8 Å resolution and belongs to space group $P2$, with unit-cell parameters $a = 50.16$, $b = 50.17$, $c = 76.60$ Å, $\beta = 99.6^\circ$. There are four molecules per asymmetric unit, with a solvent content of 35.3%.

1. Introduction

The mature seeds of mabinlang (*C. masaikai* Levl.) have a unique taste. Immediately after chewing, a bitter and astringent stimulus is left on the tongue followed by a delightful and prolonged sensation of sweetness. The mature seeds are also used as a traditional Chinese medicine. Hu and coworkers (Hu & He, 1983; Hu *et al.*, 1985) isolated two sweet proteins, named mabinlin I and II (Mab I and II), from these seeds and demonstrated that Mab II was highly heat stable with a strong sweetness property (Ding & Hu, 1986). The sweetness of Mab II was retained in solution at 353 K for at least 48 h, but Mab I lost its sweetness rapidly at 353 K. The complete amino-acid sequence determination (Liu *et al.*, 1993; Liu & Hu, 1988; Nirasawa *et al.*, 1994), the cDNA cloning and sequencing (Nirasawa *et al.*, 1996) and chemical synthesis (Kohmura & Ariyoshi, 1998) of Mab II have been carried out. We now know that Mab II is composed of an *A* chain of 33 amino-acid residues and a *B* chain of 72 amino-acid residues, with a total molecular weight of 12.4 kDa (Liu *et al.*, 1993). The *B* chain contains two intrachain disulfide bonds and connects with the *A* chain through two interchain disulfide bridges (Nirasawa *et al.*, 1993). Obviously, the next important step towards understanding the structure–function relationship of this interesting protein is to determine its three-dimensional structure. As a first step towards this, we report here the crystallization and preliminary X-ray analysis of the thermostable sweet protein mabinlin II.

Until now, six proteins, including mabinlin II, have been identified as sweet proteins: thaumatin (Van der Wel & Loeve, 1972), monellin (Morris & Cagan, 1972), mabinlin (Hu *et al.*, 1985; Liu *et al.*, 1993), curculin (Yamashita *et al.*, 1990), pentadin (Van der Wel *et al.*, 1989) and brazzein (Ming & Hellekant, 1994). Interestingly, they exhibit no significant similarities in amino-acid sequence. To date, only two crystal structures, thaumatin (Ogata *et al.*, 1992) and monellin (Somoza *et al.*, 1993), and one NMR structure, brazzein (Caldwell *et al.*, 1998), have been determined. There is also no obvious structural resemblance between the proteins. Thus, the molecular mechanism and structural basis of the sweet taste remain unknown. Therefore, more detailed structural information on sweet proteins is needed. Furthermore, sweet proteins are of great value as low-calorie sweeteners. Several of them have been produced by gene expression (Kondo *et al.*, 1997; Hahm & Batt, 1990) or by solid-phase chemical synthesis (Kohmura & Ariyoshi, 1998; Izawa *et al.*, 1996; Kohmura *et al.*, 1991). Determining the three-dimensional structures of more sweet proteins may provide clues about the sweetness determinant and may also

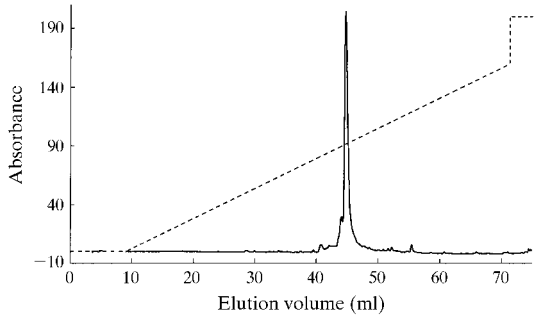

Figure 1
Chromatography of mabinlin II on an analytical reverse-phase C8 column (5 µm, 4.6 × 250 mm) on an ÄKTA Purifier system showing the high purity of the sample used in crystallization. The solvents were 0.1% TFA (buffer *A*) and 80% acetonitrile containing 0.1% TFA (buffer *B*). The protein was eluted with a linear gradient of buffer *B* (shown by the dashed line) at a flow rate of 1.0 ml min^{-1}.

lead to the production of new low molecular-weight sweeteners.

2. Experimental and results

2.1. Purification

The mabinlin II used in crystallization was purified from the mature seeds of *C. masaikai* plants grown in the south of Yunnan in China according to the method described previously (Hu *et al.*, 1985). The seed powder, defatted with petroleum ether (boiling point 303–333 K), was extracted with 50% aqueous acetone at room temperature and then precipitated at pH 10.0. After the sediment had been dissolved at pH 6.0, the sample was applied to a carboxymethylcellulose CM-52 (Whatman Inc.) column eluted with a stepwise gradient of NaCl. Mabinlin II eluted out at 0.45 *M* NaCl. After desalting, the sample used in the crystallization was obtained. When analyzed on a C8 reverse-phase column on an ÄKTA Purifier system (Amersham Pharmacia Biotech), the protein showed a high degree of purity (Fig. 1).

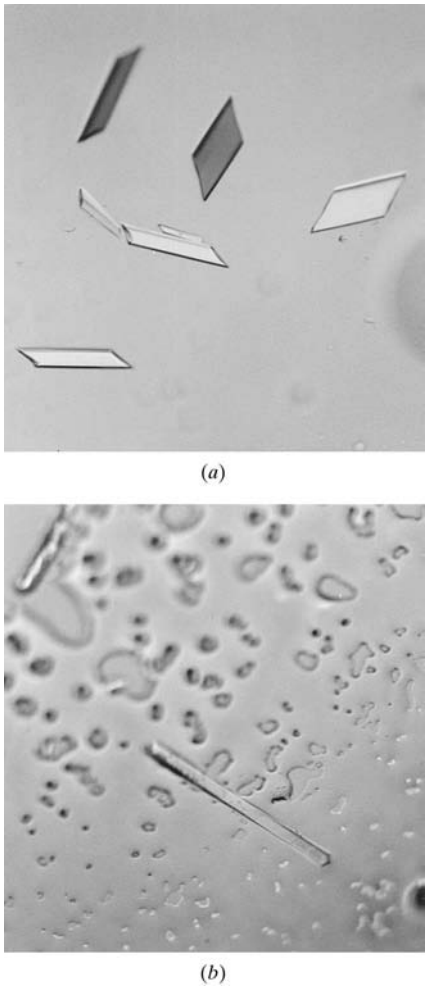

Figure 2
Crystal photographs of mabinlin II. (*a*) Crystal form I, (*b*) crystal form II.

2.2. Sweet activity

A series of solutions of mabinlin II varying in concentration were tasted by different people to test its sweetness (Kurihara & Beidler, 1969; Hu & He, 1983; Hu *et al.*, 1985). The lowest concentration inducing taste stimuli was 0.1% or 0.08 m*M*. The sweetness of 0.8 m*M* mabinlin II was equivalent to that of 0.3 *M* sucrose and its sweetness remained undiminished after incubation at 353 K for at least 48 h.

2.3. Crystallization

Crystallization experiments were performed using the hanging-drop vapour-diffusion method over a wide range of precipitants and pH values. Two crystal forms were obtained (Fig. 2). Form I crystals were obtained by mixing equal volumes of 5 mg ml^{-1} protein in 40% ammonium citrate and 0.1 *M* sodium sulfate in 0.1 *M* CHES buffer pH 9.8 containing 5% glycerol and equilibrating with the same solution. To obtain form II crystals, 10 mg ml^{-1} protein solution was mixed with an equal volume of 4.0 *M* sodium chloride, 5% dioxane in 0.1 *M* Tris buffer pH 8.5 and was equilibrated with the same solution. Form I grew to approximate dimensions of 0.02 × 0.2 × 0.3 mm after incubation at 277 K for two months and form II grew to dimensions of 0.02 × 0.02 × 0.2 mm after incubation at 295 K for more than one month.

2.4. X-ray analysis

Data from form I crystals were collected at room temperature on a MAR 345 image-plate detector mounted on an X-ray generator operating at 40 kV and 50 mA using radiation with a wavelength of 1.5418 Å. Processing of the data from form I crystals using *DENZO* (Otwinowski & Minor, 1997) showed that the crystals belonged to space group *P*2, with unit-cell parameters a = 50.16, b = 50.17, c = 76.60 Å, β = 99.6°. The crystal diffracts to 2.8 Å resolution. Assuming four molecules per asymmetric unit, V_m for crystal form I is 1.90 Å^3 Da^{-1}, with a corresponding solvent content of 35.3%, which is well within the range normally found in protein crystals (Matthews, 1968). The completeness, $R_{\rm merge}$ and number of reflections with $I > 2\sigma(I)$ in the whole data set were 81%, 0.096 and 7487, respectively. The corresponding values of the completeness and $R_{\rm merge}$ for the last resolution shell (2.90–2.80 Å) were 72% and 0.16, respectively. Since the form II crystal was too thin for X-ray diffraction using the in-house facility, we hope to study it using synchrotron radiation. Structure determination using the available data for crystal form I and attempts to grow better crystals are in progress.

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