SHORT COMMUNICATIONS

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Crystallization and preliminary X-ray analysis of D-monellin

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Abstract

D-Monellin is a chemically synthesized protein composed of all D-amino acids. It has an amino-acid sequence identical to L-monellin, a natural protein with potent sweetness. Two crystal forms of D-monellin were obtained. Both crystals were grown under conditions similiar to those used to crystallize natural L-monellin. Crystal form I has similar, but not identical, cell parameters to natural L-monellin and diffracts to 2.7 Å resolution. Crystal form II is very different and diffracts to 1.7 Å resolution using synchrotron radiation.

1. Introduction

It is well known that naturally occurring macromolecules, like proteins, have highly biased chirality. However, the reason for this has not been discovered. The fact that the constituting amino acids of D-protein are the mirror images of those of L-protein implies that the three-dimensional structure of D-protein is exactly the mirror image of L-protein. Therefore, we expected that D-monellin would crystallize into the same space group and cell parameters as L-monellin, but it did not. To understand the structural differences, if any, we plan to determine the structure of D-monellin at high resolution.

Natural monellin (L-monellin) was originally found in the berries of the West African plant *Dioscoreophyllum cumminsii* (Inglett & May, 1969), with a molecular weight of 12.4 kDa. Its sweetness potency is about 100 000 times greater than sucrose on a molar basis or 4000 times sweeter on a weight basis (Kohmura, Nio & Ariyoshi, 1992*a*,*b*), making it one of the sweetest compounds known. Natural L-monellin is a two-chain protein consisting of a 44-residue *A* chain and a 50-residue *B* chain, thus making it easier to chemically synthesize D-enantiomers with a reasonable yield. To understand the structural basis for monellin's sweetness, the structure of natural monellin has been determined (Ogata, Hatada, Tomlinson, Shin & Kim 1987) and refined (Somoza *et al.*, 1993) to 2.75 Å resolution. In addition, the structure of a genetically engineered single-chain monellin was determined to 1.7 Å resolution (Somoza *et al.*, 1993). In both crystal structures, monellin is present as a dimer with identical interactions between two monomers.

2. Materials and methods

The *A* and *B* chains of the D-monellin were synthesized by the stepwise Fmoc solid-phase method with an automated synthesizer by methods described earlier (Kohmura *et al.*, 1990, 1991, 1992*a*,*b*). The reagent, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3 tetramethyluronium hexafluorophosphate (Fields, Lloyd, Macdonald, Otteson & Noble, 1991), was used as a coupling reagent. The purity of each peptide was confirmed by analytical reverse-phase high-performance liquid chromatography, electrospray ionization mass spectrometry (ESI-MS), automated Edman degradation, and by a quantitative amino-acid analysis.

The renaturation of the *A* and *B* chains of the D-enantiomers, and subsequent purification by hydrophobic interaction chromatography (HIC) were performed as described for the preparation of monellin analogs (Kohmura *et al.*, 1992*a*,*b*). The purity of the D-protein was confirmed by HIC, ESI-MS, and by a quantitative amino-acid analysis. In contrast to natural L-monellin, synthetic D-monellin was devoid of any sweetness and was essentially tasteless when tasted as a lyophilized powder (0.1 mg).

D-Monellin has the opposite optical activity of L-monellin when tested with circular dichroism (Ariyoshi & Kohmura, 1994), suggesting the possibility that it is the mirror image of L-monellin. The optimum crystallization conditions were slightly different from those for natural L-monellin. A solution containing 5 mg ml^{-1} synthetic D-monellin, 10 m*M* sodium phosphate buffer (pH 7.2) and 14% PEG 8000 was equilibrated for several days against a solution of 20 m*M* sodium phosphate buffer (pH 7.2), 28% PEG 8000 at 277 K. Crystals appeared only after raising the reservoir concentration to 40% PEG 8000. [Natural L-monellin crystallizes from a solution of 6 mg ml^{-1} protein, 10 m*M* sodium phosphate buffer (pH 7.2), and 6% PEG 8000 equilibrated against 33.3% PEG 8000.] Two crystal forms of D-monellin were observed. The first one (crystal form I) is thick, plate-like, and belongs to space group $P2_1$, with unit-cell parameters $a = 39.9$, $b = 84.8$, $c = 71.9$ Å, $\alpha = \beta = 90.0$, $\gamma = 99.9°$ which are similar, but not identical, to those of natural L-monellin [*cf.* natural monellin in $P2_1$ space group with $a = 39.8$, $b = 87.2$, $c = 72.1$ Å, $\gamma = 107.3°$ (Somoza *et al.*, 1993)]. Later, rod-like crystals (crystal form II) formed under the same conditions. The unit-cell parameters are $a = 40.59$, $b = 33.03$, $c = 41.35$ Å, $\alpha = \gamma = 90.0$, $\beta = 96.43°$ with two molecules in the unit cell of space group $P2$ or $P2_1$. The near equivalence of the *a* and *c* axes led us to investigate other possible unit cells and space groups. These were rejected by us on the basis of crystal packing and intensity statistics. The typical crystal size observed is 300 × 150 × 80 µm for crystal form I and 400 × 50 × 40 µm for crystal form II.

Crystal form I initally diffracted to 2.7 Å resolution and degraded to 4 Å resolution rapidly on an R-AXIS imaging-plate detection system with a Cu $K\alpha$ rotating anode source at 277 K. Attempts to flash-freeze this form have not been successful. Crystal form II diffracted to 1.7 Å resolution at beamline VII-1 of the Stanford Synchrotron Radiation Laboratory (SSRL),

using an X-ray wavelength of 1.08 Å. A complete data set for crystal form II was collected from one crystal at 277 K on a MAR imaging plate. The data set was processed using the program *DENZO* (Otwinowski, 1993) and integrated by *SCALEPAK* in space group *P*2. A total of 18 438 unique reflections encompass 95% of data completeness with an R_{merge} [$R_{merge} = \sum |I(h) - \langle I(h)\rangle| / \sum I(h)$, with $I(h)$, observed intensity and $\langle I(h)\rangle$, mean intensity of reflection h over all measurement of $I(h)$] of 4.5% for all reflections between 30 and 1.8 Å.

Crystal form I has four molecules in an asymmetric unit and the calculated V_m value is 2.40 $Å^3\ Da^{-1}$ corresponding to a solvent content of 49%. The cell volume of crystal form II is approximately 1/4 that of natural L-monellin suggesting that there is one molecule per asymmetric unit. This corresponds to a V_m value of 2.20 $Å^3\ Da^{-1}$ and a solvent content of 44%.

Initial attempts to solve the structure of D-monellin by molecular replacement using the mirror image of L-monellin structure have been unsuccessful for both crystal forms, suggesting that the D-monellin structure has a different oligomeric structure and/or a sufficiently different monomeric structure. To understand this unusual and unexpected observation we plan to solve the structure using other methods of structure determination.

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