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Refolding the sweet-tasting protein thaumatin II from insoluble inclusion bodies synthesised in *Escherichia coli*

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Abstract

A synthetic gene encoding the amino acid sequence of the strongly sweet-tasting protein thaumatin II has been expressed in *Escherichia coli*. The recombinant protein has been renatured from inclusion bodies using a reduced/oxidized glutathione system to yield a purified protein preparation that is indistinguishable from native thaumatin with respect to its biochemical, spectroscopic and organoleptic properties.

Keywords: Thaumatin; Sweet taste; Protein refolding; Thiol/disulfide redox

1. Introduction

Thaumatin is an intensely sweet protein (100,000 times sweeter than sucrose on a molar basis) which has been approved for human consumption, and is currently used in products such as chewing gum, dairy produce and pharmaceuticals (Etheridge, 1994; Van der Wel & Loeve, 1972; Witty, 1998). There are at least five forms of thaumatin which occur naturally in the arils of the fruit of the West African plant *Thaumatococcus danielli* Benth. The two predominant forms are thaumatin I and II which both taste sweet (Van der Wel & Loeve, 1972). These two forms are likely to adopt similar structures and as a consequence are likely to present a similar sweet glucophore, since they differ at only six positions over a single-chain polypeptide of 207 amino acids (de Vos, Hatada, Van der Wel, Krabbendam, Peerdeman & Kim, 1985; Ogata, Gordon, de Vos & Kim, 1992).

Although natural thaumatin is commercially prepared as a sweetener, the availability of the fruit from which it is extracted and the costs of collection have limited its industrial use. There have been several attempts to produce thaumatin in microbial fermentation through the use of recombinant DNA technology. These have met with mixed rates of success, either due to poor protein yields or the production of organoleptically inactive protein (Edens, Born et al., 1984; Edens, Heslinga et al., 1982; Edens & Van der Wel, 1985; Hahm & Batt, 1990; Faus, Patino, del Rio, del Moral, Sisniega & Rubio, 1996; Faus, del Moral et al., 1998; Illingworth, Larson & Hellekant, 1988,1989; Zemanek & Wasserman, 1995). However, more recently, acceptable yields of a sweet tasting recombinant product have been reported from the filamentous fungus *Aspergillus awamori* (Moralejo, Cardoza, Gutierrez & Martin, 1999). The success of this approach not with-standing, we saw the expression of relatively high-levels of thaumatin II in *E. coli* in the form of insoluble inclusion bodies (Faus et al., 1996) as an opportunity to study the constraints on the folding of the protein to a biologically active conformation in vitro. Natural thaumatin contains eight intramolecular disulfide bonds (and no free cysteines) (Van der Wel, Iyengar, Van Brouwershaven, Wassenaar, Bel & Van der Ouderaa, 1984), all of which must be aligned in register before a soluble sweet-tasting product can be achieved.

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2. Materials and methods

2.1. Purification of native thaumatin

Thaumatin was purified from crude thaumatin by SP-Sephadex (Pharmacia, Uppsala, Sweden) ion-exchange column chromatography (Van der Wel & Loeve, 1972) and Sephadex G-75 (Pharmacia) gel filtration column chromatography. The resulting thaumatin sample gave a single band on both sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and non-denaturing polyacrylamide gel electrophoresis. Purified thaumatin was used as reference material for the chemical and organoleptic properties of the recombinant thaumatin.

The protein concentration of native thaumatin was measured spectrophotometrically at 278 nm, based on an extinction coefficient at 1% of $E = 7.69$ (Van der Wel & Loeve, 1972). For denatured forms of thaumatin, the protein concentration was determined using the BCA reagent (bicinchoninic acid) according to the manufacturer's instruction (Pierce, USA) with bovine serum albumin standard controls.

2.2. Antibody preparation and detection

Antibody to thaumatin was produced by inoculation of the protein into adult Dutch Half Lop rabbits using Freund's adjuvants according to standard procedures. Western blotting was carried out using a Bio-Rad mini-transblot system according to the manufacturer's instructions with Problot membranes (Applied Biosystems, UK). Bound antibodies were detected using anti-rabbit IgG (Fc fragment conjugated to alkaline phosphatase; Life Technologies, UK) and visualised with nitro blue tetrazolium (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma).

2.3. Expression of recombinant thaumatin II in E. coli

A synthetic gene encoding thaumatin II was expressed from the T7-inducible vector pET-8c in BL21(DE3)-pLysS *E. coli* cells, as described previously (Faus et al., 1996). Routinely frozen stocks of an established thaumatin-expressing culture were used to inoculate 2×250 ml conical flasks containing 50 ml LB culture medium plus 50 μg ml^{-1} ampicillin and 30 μg ml^{-1} chloramphenicol to select for the pET-8c and pLysS plasmids (Studier, 1991), respectively. The cultures were incubated for 8 h, shaking at 37°C and used to inoculate 500 ml of the same medium in 2 l flasks. These cultures were incubated at 30°C with shaking until $O.D._{600} = 0.6$ (ca. 2–3 h). One milliliter samples of the pre-induction control cultures were collected before adding the inducer isopropyl-β-D-thiogalactopyranoside to each culture to a final concentration of 0.4 mM, and incubating for a further 4 h with shaking at 30°C. After 4 h induction, 1 ml of the induced culture was removed; thaumatin expression was monitored by the appearance of the characteristic protein band at 21 kDa upon separation of the total cell proteins by 12.5% SDS-PAGE. For N-terminal sequence analysis, these gels were electroblotted onto Immobilon P (Millipore, USA) and analysed by automated Edman degradation on an ABI 410C protein sequencer (Applied Biosystems). The cells were harvested by centrifugation at 3000 × g for 4 min at 4°C. Batch yields were assessed on the weight of each initial cell pellet. Cell pellets could then be stored up to 3 days at 4°C until required.

2.4. Purification of recombinant thaumatin

Cell pellets were resuspended at approximately 4 ml g^{-1} of pellet in 20 mM phosphate buffer pH 7.0, 0.5 mM phenylmethanesulfonyl fluoride, 1 mM EDTA, and disrupted by two passages in a French Press at 1000 psi. Insoluble materials, including the inclusion bodies, were then collected by centrifugation at 45,000 × g for 15 min at 4°C. These pellets were then washed by resuspension in 10 ml of 100 mM Tris/Acetate pH 8.6, 100 mM NaCl, 10 mM EDTA, 0.5% Triton X-100. The inclusion bodies were then re-pelleted at 45000 × g for 15 min at 4°C before solubilisation in 5 ml of buffer (solubilisation buffer contains 100 mM Tris/Acetate, pH 8.6, 6 M guanidine-HCl, 1 mM EDTA and 1 mM 2-mercaptoethanol). Any remaining insoluble materials were removed by filtration through a 0.22 μm filter before loading on to a Superose-12 column (Pharmacia) pre-equilibrated with solubilisation buffer at the relatively slow flow rate of 0.5 ml min^{-1}, to prevent back pressure. Collecting 1 ml fractions, a peak around fractions 12 and 13 generally contained thaumatin as ascertained by SDS-PAGE (21 kDa).

The thaumatin-containing fractions were refolded by slow dilution over 16 h at room temperature into 1 l of stirred phosphate-buffered saline (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, pH 7.2), containing oxidized (0.306 gl^{-1}) and reduced (0.307 gl^{-1}) glutathiones. The protein was concentrated over an Amicon, filter (10 kDa cut-off) and further purified by FPLC (Pharmacia) using an SP-Sephadex column developed with a 0.2–1.0 M linear gradient of NaCl.

2.5. Fluorescence spectroscopy

Fluorescence spectra were obtained using a Perkin-Elmer LS-50B fluorimeter. Protein tryptophan emission spectra were determined at 20°C between 305 and 400 nm with an excitation wavelength of 296 nm. Corresponding blank solutions were used to correct for Raman scattering by solvent.

The solubilised recombinant thaumatin was also refolded in the presence of 100 μM ANS (anilinonaphthalene-8-sulfonate) to monitor the change in hydrophobicity. The fluorescence intensity of ANS was recorded using a Perkin-Elmer LS.50 fluorescence spectrophotometer (exitation at 355 nm emission at 470 nm) and corrected with respect to the protein concentrations.

2.6. Circular dichroism spectroscopy (CD)

CD spectra were recorded at 20°C using a Jobin-Yvon CD6 spectropolarimeter. To obtain far UV spectra, a pathlength of 0.01 cm at a protein concentration of 0.5 mg/ml^{-1} was used. Protein secondary structure analysis was by the CONTIN method (Provencher & Glöckner, 1981) and the method of Perczel, Hollósi, Tusnády & Fasman (1991).

2.7. Toxicity testing of recombinant thaumatin

Acute oral toxicity testing of the recombinant thaumatin was performed according to standard international guidelines using six 'Swiss' strain mice ["OECD Guideline for the testing of chemicals" — acute oral toxicity — acute toxic class method (Section 4, Number 423)].

2.8. Sensory evaluation of the sweetness of thaumatin

Five millilitres of test solution were dispensed into disposable polyethylene-coated paper cups kept at room temperature. The protein concentrations (10–500 nM) of the test solutions were made up with 5 mM phosphate buffer, pH 7.2. Initially a panel consisting of 8 volunteers was asked to determine the thresholds of sweetness for the thaumatin preparations by tasting sample solutions from the lowest to the highest concentrations. The panel was then asked to decide on the relative sweetness of random series of sample solutions containing a range of thaumatin concentrations against unmarked reference solutions of sucrose (0.05–0.5 M). After tasting each sample, the panellists were asked to spit-out the solution and were provided with distilled water to thoroughly rinse their palates until no aftertaste was detected.

3. Results and discussion

3.1. Analysis of recombinant thaumatin

Like many polypeptides of eukaryotic origin, thaumatin is synthesised in *E. coli* as an insoluble inclusion body. The SDS-PAGE shown in Fig. 1A demonstrates the induction of the synthetic gene product in response to IPTG, as a 21 kDa protein band of similar size to native thaumatin. The *E. coli* cells carrying the recombinant protein were sheared by French press to release the inclusion bodies, which could then be isolated by centrifugation. Fig. 1A shows that neither the supernatant nor a Triton-X100 wash of the pellet contained appreciable thaumatin. To ensure that the observed protein was due to the expression of the thaumatin polypeptide, the 21 kDa band was separated by SDS-PAGE, electro-blotted and N-terminally sequenced. The resulting sequence ATFEI corresponds to the translation of the synthetic gene less the initiating methionine, which is presumably removed by *E. coli*. This sequence precisely matches the mature N-terminal sequence of native thaumatin (Iyengar et al., 1979). Sections of these Western blots were also used for immunodetection with antibody raised against thaumatin. Fig. 1B shows that the anti-thaumatin antibody clearly recognises the prominent 21 kDa band synthesised in *E. coli* as well as several residual higher and lower molecular weight forms, which are all but absent in the pre-induced control.

The inclusion bodies were solubilised in 6 M guanidine-HCl in the presence of excess 2-mercaptoethanol as reducing agent. This material was further purified by gel filtration on a Superose-12 column and the polypeptide fractions containing the 21 kDa band collected and pooled. Slow dialysis of these fractions prevented re-precipitation. These fractions continue to show residual higher molecular weight protein forms on SDS-PAGE which are recognisable by the anti-thaumatin antibody (Figs. 1A and B). These forms of the protein are more evident when run on SDS-PAGE in the absence of the reducing agent 2-mercaptoethanol (Fig. 1C). It is, therefore, likely that they arise due to incomplete reduction of the disulfides prior to electrophoresis and the inclusion bodies represent a series of mixed inter and intra-molecular protein disulfides, a condition common to many recombinant proteins expressed in *E. coli* which feature cysteine residues. This form of the protein was not noticeably sweet-tasting.

The thaumatin-containing fractions were, therefore, refolded by slow dilution in the presence of mixed disulfides, generated by oxidized and reduced glutathiones. The dilute protein was concentrated and purified further by ion-exchange chromatography. This purified protein produced a single band upon reducing and non-reducing SDS-PAGE, similarly to that of purified native thaumatin (Figs. 1A and C). This purified recombinant protein was found to taste sweet. Using this methodology, approximately 40 mg of purified thaumatin could be produced per litre of batch culture, which equates to a relatively low recovery of 10% of the initial inclusion body protein.

Native thaumatins I and II are subject to the reversible loss of sweetness following mild thermal or pH-driven

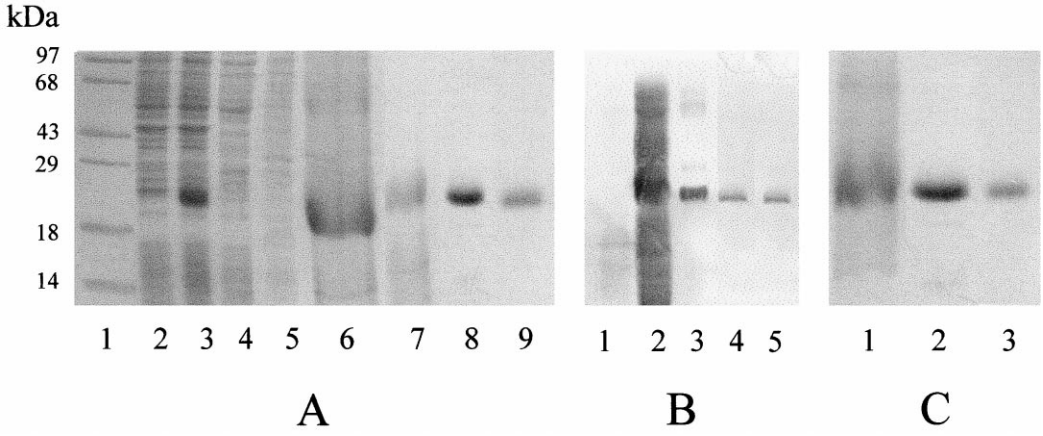

Fig. 1. (A) SDS-polyacrylamide gel electrophoresis of reduced (2-mercaptoethanol) proteins. Lane 1, molecular weight markers (14-97 kDa); lane 2, total cell lysate of *E. coli* BL21(DE3) pLysS pET-8c carrying synthetic thaumatin pre-IPTG induction; lane 3, post-IPTG induction of the same; lane 4, cytosolic fraction post-IPTG induction; lane 5, Triton X-100 wash of the inclusion bodies; lane 6, solubilised inclusion bodies; lane 7 inclusion bodies post Superose-12 gel filtration; lane 8, refolded recombinant thaumatin II following ion-exchange chromatography; lane 9, purified native thaumatin control. (B) Western blot immuno-detected with rabbit anti-thaumatin. Lane 1, total cell lysate of *E. coli* BL21(DE3) pLysS pET-8c carrying synthetic thaumatin pre-IPTG induction; lane 2, post-IPTG induction of the same; lane 3, solubilised inclusion bodies post Superose-12 gel filtration; lane 4, refolded recombinant thaumatin II following ion-exchange chromatography; lane 5, purified native thaumatin control. (C) SDS-polyacrylamide gel electrophoresis of non-reduced proteins. Lane 1, solubilised inclusion bodies post Superose-12 gel filtration; lane 2, refolded recombinant thaumatin II following ion-exchange chromatography; lane 3, purified native thaumatin control.

denaturation (Van der Wel & Loeve, 1972). Similarly, the recombinant form of the protein reported here can be refolded to an active conformation. However, more severe treatment of thaumatin results in an irreversible change leading to precipitation. This change is due to the formation of intermolecular disulfide linkages, which are subject to irreversible chemical modification (Kaneko & Kitabatake, 1999). Labile disulfide bonds have a major influence on stability of the secondary structure of this protein (Van der Wel et al., 1984; Van der Wel, 1994) and its ability to be refolded as recombinant protein product.

3.2. Fluorescence spectroscopy

Using an excitation wavelength of 296 nm, the environment of the tryptophan residues within the protein could be examined by fluorescence spectroscopy. Changes in the local environment of these residues often cause shifts in the emission maxima, such that a buried residue will peak around 325 nm and solvent-exposed residues will be shifted to 350 nm. Native thaumatin contains two tryptophan residues that produce emission maxima at 335 nm and 342 nm, indicating varying degrees of solvent exposure of the residues. As evidence of folding, the recombinant protein experienced a shift in the fluorescence spectrum from denaturing solution (6 M guanidine-HCl) with the loss of a peak at 355 nm.

The solubilised recombinant thaumatin was also refolded in the presence of the fluorescent reagent ANS, which binds hydrophobic exposed regions of the protein to produce a fluorescence signal at 470 nm when excited at 355 nm. The intensity of this signal was found to fall during the refolding procedure, providing further evidence that the protein was undergoing a change in conformation.

3.3. Circular dichroism

CD signals in the far UV (180–250 nm) provide information on the peptide bonds and secondary structures of proteins. These signals are often used to monitor structural changes in proteins, and in particular the gain or loss of secondary structure due to folding/unfolding events. The far UV spectrum of the refolded recombinant thaumatin II is presented in Fig. 2. The spectra of the recombinant and native proteins are very similar as found previously for the native thaumatin I and II proteins (Korver, Gorkom & Van der Wel,

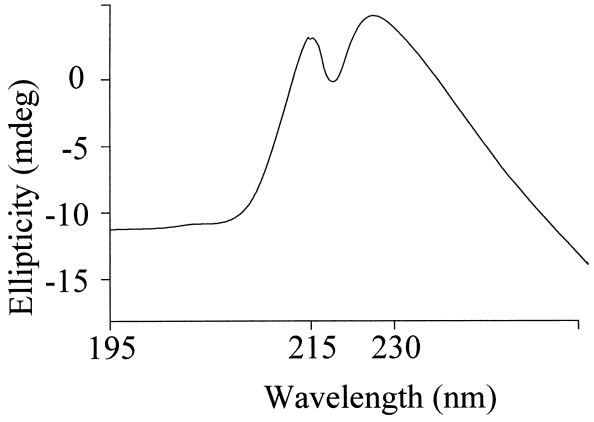

Fig. 2. The far UV CD spectrum of refolded recombinant thaumatin II at pH 7.2 and 20°C.

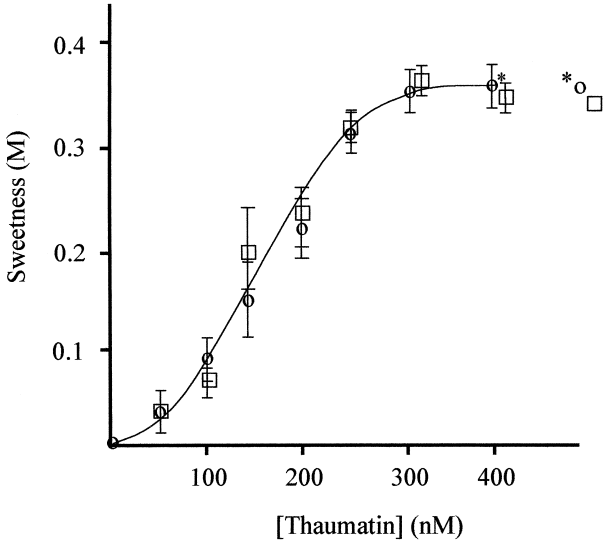

Fig. 3. The sweet intensity of recombinant thaumatin II compared to native thaumatin. The sweet intensity data for the purified native (○) and recombinant (□) proteins are plotted as function of concentration. The relative sweetness is evaluated against reference sucrose solutions at pH 7.2 in 5 mM phosphate buffer. The data points represent the mean values ±S.E. of the data from the 8 subjects. The asterisks indicate the concentrations at which the persistence of the sweet taste was perceived.

1973). The spectrum clearly demonstrates that the recombinant protein adopts a secondary structure, calculation of which, based on the CONTIN method (Provencher & Glöckner, 1981) indicates a constitution of 9% α-helix and 42% β-sheet. These structure calculations are consistent with the three-dimensional structures, as determined by X-ray crystallography (de Vos et al., 1985; Ogata et al., 1992).

3.4. Acute toxicity test

Before sensory trials could begin, acute toxicity tests on mice, of the purified recombinant thaumatin, were performed. Six 'Swiss' strain mice were given, orally, up to 24 mg of recombinant thaumatin per kg body weight, representing over 400-times more than that used in human foods. Over a 14-day period these mice remained healthy without any change in behaviour. Following autopsy, all tissues were found to be normal. By this criterion, the recombinant protein preparation was considered to be non-toxic.

3.5. Sensory analysis

Initial experiments determined the threshold of detection of the sweet taste of the recombinant protein in human volunteers to be around 50 nM, as found for the native protein tested in parallel, and as has been previously recorded for native thaumatins I and II (Van der Wel & Loeve, 1972). The volunteers were also asked to compare qualities of the sweet taste and the persistence of the sensation between random paired samples containing native or recombinant thaumatin at the same concentration. The volunteers considered there to be no differences in the perception of the two solutions. At a later date the panel of volunteers were asked to decide on the relative sweetness of a random series of solutions containing a range of thaumatin concentrations against unmarked reference solutions of sucrose (0.05–0.5 M). These data are presented in Fig. 3, where the concentrations of the recombinant and control thaumatin preparations show similar thresholds and saturation points for the sweet taste when considered as functions of the protein concentration. Under these conditions, the saturation level for both proteins is around 300 nM, which is approximately equivalent to 0.35 M sucrose, making the thaumatin preparations in the order of 10^5-times sweeter than sucrose on a molar basis. This value is consistent with previous estimates of the relative sweetness of native thaumatins (Van der Wel & Loeve, 1972). The volunteers perceived no differences in the quality of the sweet taste of the two thaumatin preparations. However, by the same criterion, all the volunteers noted the persistence of the sweet taste and the sensation of a liquorice after-taste at saturating concentrations of both proteins. Taken together, these data suggest that the recombinant protein is functionally equivalent to the natural protein.

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