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Bacterial expression, purification and preliminary X-ray crystallographic characterization of the invertase inhibitor Nt-CIF from tobacco

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Acid invertases catalyzing the breakdown of sucrose are regulated at the post-translational level by extracellular inhibitory proteins of 16–20 kDa molecular weight in a pH-dependent manner. Little is known about the characteristics of the underlying protein–protein interaction. Here, the expression, purification, characterization, crystallization and initial X-ray analysis of a biologically active invertase inhibitor Nt-CIF from tobacco is reported. Four crystal forms covering a wide pH range have been obtained and data sets at resolutions higher than 2.5 Å have been collected.

1. Introduction

Invertases (EC 3.2.1.26) are plant-specific enzymes that catalyze the irreversible hydrolysis of sucrose, the predominant transport form of assimilated carbon in plants. They are major players in metabolic and developmental processes and exist in compartment-specific isoforms within the cytosol, the vacuole and the extracellular space. Vacuolar and extracellular acid invertases are evolutionarily related. They show a K_M for sucrose in the millimolar range and pH optima between pH 4.5 and 5 (Sturm, 1999). At the post-translational level, these 'extracytosolic' enzymes may be regulated by inhibitory proteins of 16–20 kDa molecular weight. In the case of tobacco, an extracellular invertase inhibitor termed Nt-CIF has been characterized as a non-glycosylated monomer that appeared to be tightly co-localized with cell-wall invertase and to affect its activity in a pH-dependent manner. Moreover, the inhibition could be modulated by sucrose and divalent cations in the millimolar range (Weil *et al.*, 1994). The inhibitor, which shares characteristics with other proteins (Matsushita & Uritani, 1976; Pressey, 1966), was cloned, recombinantly overexpressed and purified using refolding protocols (Greiner *et al.*, 1998), but with limited yield.

The biotechnological relevance of invertase inhibitors has been pointed out in the case of the cold-induced sweetening of potato tubers, which is a major food-storage problem. The expression of an invertase inhibitor from tobacco in the tubers could reduce the accumulation of free hexoses, making inhibitor–invertase interaction an attractive target for interfering with the sweetening process (Greiner *et al.*, 1999).

Furthermore, Camardella and coworkers have pointed out the sequence homology between invertase inhibitors and a pectin methylesterase inhibitor from kiwi, which shares four conserved cysteine residues that are involved in the formation of two disulfide bonds in the kiwi protein (Camardella *et al.*, 2000). Pectin methylesterases hydrolyze pectin methylester groups, producing pectin with a lower methylation degree and methanol. The regulation of ethanol and methanol concentration in fruits has been ascribed to these enzymes (Frenkel *et al.*, 1998). However, two recent X-ray structures of bacterial (Jenkins *et al.*, 2001) and plant (Johansson *et al.*, 2002) pectin methylesterases gave no insight into the regulation of these enzymes.

Interestingly, the recent identification of a new type of allergen in plane-tree pollen sharing sequence homology with invertase inhibitors (27% identity with Nt-CIF; Asturias *et al.*, 2003) further enlarges the inhibitory familiy.

Currently, the specific and highly regulated protein–protein interaction involving invertase inhibitors and acid invertases as well as pectin methylesterases and their cognate inhibitors has not been studied in detail. Recent evidence has confirmed that individual members of the novel inhibitor family react either with pectin methylesterase or with invertase but never with both (Scognamiglio *et al.*, 2003). The structural basis for this specificity is not known. Here, we report the recombinant overexpression of Nt-CIF in *Escherichia coli*, using a thioredoxin fusion system in Origami cells, followed by high-yield purification and crystallization in four different lattices. In addition, we demonstrate biological activity in invertase-inhibition assays. Our studies now make invertase inhibitors accessible to

detailed structural and biochemical analysis.

2. Materials and methods

2.1. Cloning

Nt-CIF was PCR-amplified using sense primer 5′-TAT ATC CAT GGG CAA TAA TCT AGT AGA AAC TAC ATG-3′, antisense primer 3′-ATA CAT TTA TGC GGC CGC TAC AAT AAA TTT CTG ACA ATA-5′ and a pBluescript SKII vector (Stratagene) containing the Nt-CIF gene as template (Greiner *et al.*, 1998). Subsequently, the *Nco*1/*Not*1-excised Nt-CIF fragment was cloned into a modified pET21d vector (Novagen) providing thioredoxin A (trxA) followed by a 6×His tag (amplified from pET32a; Novagen) and a tobacco etch virus (TEV) protease-cleavage site as an N-terminal fusion partner (pETM 20) (for details, see http://www.embl-heidelberg.de/ExternalInfo/geerlof/draft_frames/index.html).

2.2. Protein expression and purification

The fusion protein was expressed in *E. coli* Origami (DE3) from Novagen. Cells grown to an $OD_{600\,nm}$ of 0.8 in Terrific Broth (Sambrook & Russell, 2001) were induced for 26 h at 289 K with 0.2 m*M* isopropyl-β-D-galactopyranoside (IPTG). 40 g of pelleted cells were resuspended in buffer *A* (50 m*M* Na_2HPO_4/NaH_2PO_4 pH 7.5, 300 m*M* NaCl, 100 m*M* sucrose, 15 m*M* imidazole, 1% Triton X-100) and lysed with an EmulsiFlex C5 (Avestin). The suspension was centrifuged at 30 000*g* for 1.5 h and the supernatant applied to a C10/10 column (Amersham Biosciences) containing 8 ml Chelating Sepharose Fast Flow resin (Amersham Biosciences) preloaded with 100 m*M* $NiSO_4$. The column was washed with buffer *A* followed by the same buffer lacking Triton X-100. Finally, the protein was eluted on a gradient against buffer *B* (50 m*M* Na_2HPO_4/NaH_2PO_4 pH 7.5, 300 m*M* NaCl, 100 m*M* sucrose, 250 m*M* imidazole). The fusion protein was isolated by gel-filtration chromatography (HiLoad 16/60 Superdex 75 prep-grade, Amersham Biosciences), concentrated to 3 mg ml^{-1} and cleaved for 3 h at 303 K with recombinant 6×His-tagged TEV protease. The untagged Nt-CIF protein was separated from trxA and TEV in a second metal-affinity step and concentrated by dialysis against dry polyethylene glycol (PEG) 20 000 in finger-shaped dialysis vials (Schleicher & Schüll). Finally, the protein was dialyzed against 10 m*M* HEPES pH 7.0, 150 m*M* NaCl using 3500 molecular-weight cutoff Slide-A-Lyzer dialysis cassettes (Perbio Science). Protein concentrations were estimated from the optical density at 280 nm using the extinction coefficient and the molecular weight calculated from the Nt-CIF protein sequence with *ProtParam* (http://us.expasy.org/tools/protparam.html).

2.3. Activity assay of inhibitor function

Tobacco cell-wall invertase has been prepared from cell-suspension cultures as previously described (Weil *et al.*, 1994). Inhibition assays of invertase activity were performed following previously described protocols (Weil & Rausch, 1994).

2.4. Crystallization and X-ray analysis

Crystals were grown at room temperature by vapour diffusion from hanging drops composed of equal volumes (typically 1 µl plus 1 µl) of protein solution (at about 12 mg ml^{-1}) and crystallization buffer suspended over 0.5 ml of the latter as reservoir solution. Crystals were initially obtained using Crystal Screen, Crystal Screen 2 and PEG/Ion Screen (Hampton Research). Subsequently, conditions were refined by adjustment of pH and precipitant concentration. Crystals appeared after 10 h to 3 d and grew to their final size (see Fig. 1) within a week.

For X-ray analysis, crystals were transferred to a reservoir solution containing 5%(*v*/*v*) glycerol, which served as a cryoprotectant for all crystal forms described, and flash-frozen in liquid nitrogen. Data collection under cryogenic conditions (100 K, Oxford Cryosystems Cryostream) yielded a data set for crystal form *A* collected at beamline ID29 (ADSC Q210 two-dimensional detector) of the European Synchrotron Radiation Facility (ESRF), Grenoble, France. Another data set (form *B*) was recorded at beamline BW7A (MAR 165 mm CCD detector) of EMBL c/o DESY, Hamburg, Germany. Finally, data for crystal forms *C* and *D* were acquired at ESRF beamline ID14-4 (ADSC Q4R CCD

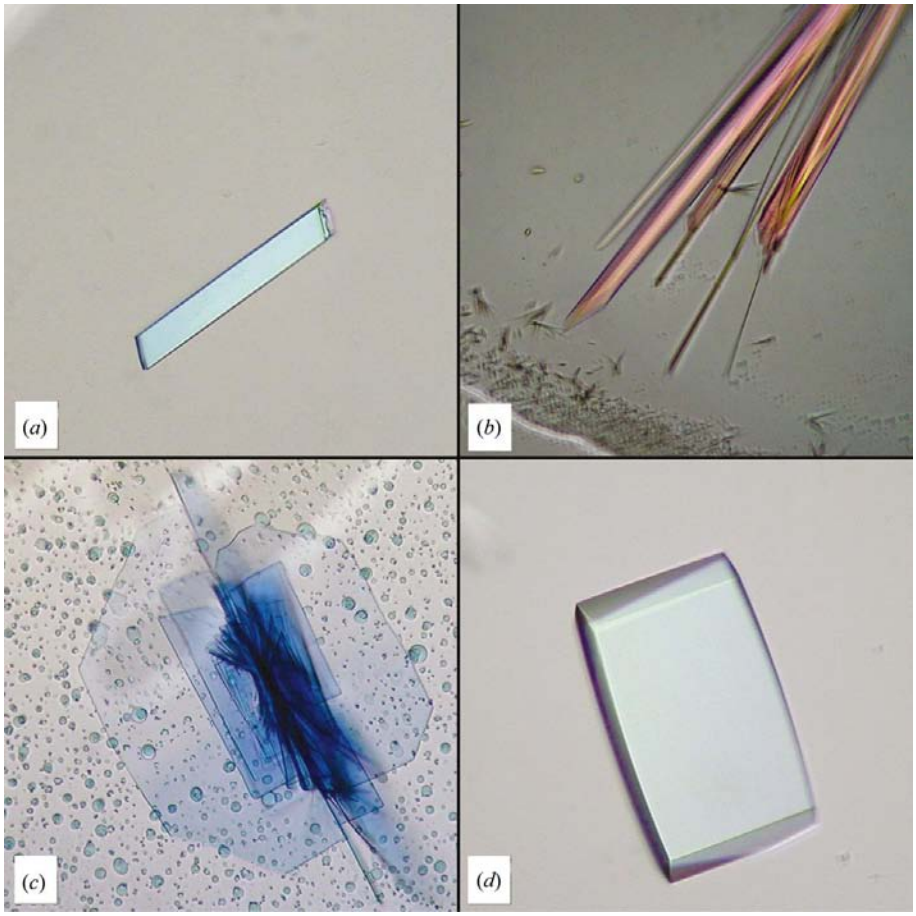

Figure 1
Pictures of Nt-CIF crystal forms observed in hanging-drop vapour-diffusion experiments. (*a*) Form *A*, about 50 × 50 × 400 µm in size, (*b*) sword-like needles (form *B*) growing throughout the drop (40 × 40 × 2000 µm), (*c*) form *C*, about 30 × 1000 × 1000 µm, shown after staining with Izit crystal dye (Hampton Research), (*d*) single crystal (form *D*) grown in the presence of $CdCl_2$, average size 100 × 200 × 350 µm.

detector). Data processing and scaling were performed with the program *XDS* (version December 2002; Kabsch, 1993) (see Table 1).

3. Results and discussion

In initial trials of bacterial expression, we found the inhibitor in inclusion bodies. Refolding protocols (Greiner *et al.*, 1998) did not yield protein in satisfactory amounts to perform detailed biochemical and structural analysis. The expected presence of disulfide bridges (Camardella *et al.*, 2000) prompted us to employ fusion to thioredoxin A, combined with expression in *E. coli* Origami (Novagen) (Bessette *et al.*, 1999). The protein was purified using two metal-affinity steps interspersed by size-exclusion chromatography to overcome problems in the separation of Nt-CIF from trxA, as they are similar in both size and charge. In our hands, the inhibitor could only be concentrated by dialysis against polyethylene glycol 20 000. The final yield of the protein preparation has been estimated to be 0.5 mg of pure inhibitor per 1 g cell pellet. After concentration to approximately 12 mg ml^{-1} the protein was desalted and flash-frozen in liquid nitrogen before storage at 203 K.

The purified inhibitor, which shared the N- and C-terminus with the mature protein *in planta* (residues 20–166), has been demonstrated to be biologically active in test assays monitoring the activity of invertase purified from tobacco-cell suspension cultures (Fig. 2). The inhibitory effect is about tenfold stronger than observed in inhibition studies using the refolded protein (Greiner *et al.*, 1998) at a comparable protein concentration.

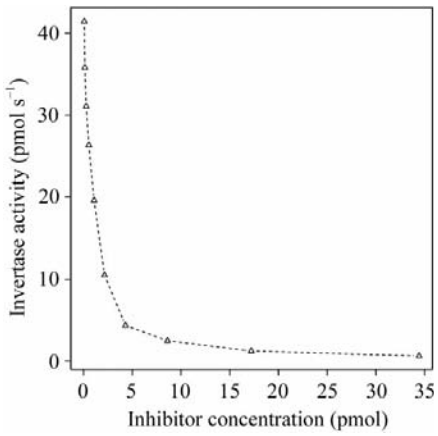

Figure 2
Invertase-activity inhibition assay using a cell-wall invertase purified from tobacco-cell suspension cultures and the purified inhibitor. The rate of sucrose hydrolysis in the presence and absence of Nt-CIF is monitored.

Table 1
Summary of crystallization and preliminary X-ray analysis.

Values in parentheses give values for the highest resolution shell.

Crystal form	*A*	*B*	*C*	*D*
Crystallization				
Precipitant	17% PEG 4K	25% PEG 4K	4.0 *M* Na formate	4.0 *M* Na formate
Buffer	0.1 *M* NaOAc pH 4.6	0.1 *M* bis-tris pH 9.0	0.1 *M* bis-tris pH 7.0	0.1 *M* bis-tris pH 7.0
Salt	0.2 *M* Li_2SO_4	0.2 *M* NaI		
Additive				30 m*M* $CdCl_2$
Preliminary analysis				
Resolution limit† (Å)			<2	<2.5
Lattice type			oC	oP
Unit-cell parameters (Å)				
a			60	59
b			106	96
c			56	126
Data collection				
Resolution limit† (Å)	1.78	2.4		
Space group	$P222_1$	$P2_12_12_1$		
Unit-cell parameters (Å)				
a	47.16	40.03		
b	57.43	50.21		
c	131.29	131.31		
Resolution of data set (Å)	15–1.78 (1.9–1.78)	15–2.45 (2.54–2.45)		
No. unique reflections	34522 (5977)	10876 (1025)		
Redundancy	7.7 (7.6)	5.5 (5.5)		
$\langle I/\sigma(I)\rangle$	13.8 (6.5)	11.2 (4.55)		
R_{sym}‡ (%)	11.0 (34.1)	9.9 (34.1)		
Completeness (%)	98.5 (97.6)	99.6 (99.7)		
V_M§ (Å^3 Da^{-1})	1.90	2.08		

† λ was 0.934 Å for all diffraction data recorded. ‡ As defined in *XDS* (Kabsch, 1993). § Assuming three and two monomers per asymmetric unit, respectively (Matthews, 1968).

Four crystal forms grown under conditions covering a pH range between 4.6 and 9 have been obtained (Fig. 1) from hanging drops (see §2) incubated at room temperature with PEG 4000 (forms *A* and *B*) or sodium formate (form *C* and *D*) as precipitant. Crystallization conditions are summarized in Table 1. Obtaining single crystals for X-ray crystallographic analysis usually required several trial experiments. Form *D* crystals grew in the presence of $CdCl_2$ and initially only appeared after streak-seeding in pre-equilibrated drops set for the form *C* species in an additive screen (Hampton Research). All crystal forms show high diffraction potential, with resolution limits between 1.7 and 2.5 Å. Data sets have been collected for form *A* and *B* crystals at ESRF or EMBL c/o DESY (see Table 1). Calculation of the Matthews parameter (see Table 1) along with self-rotation studies in *POLARRFN* (Collaborative Computational Project, Number 4, 1994) suggest three monomers per asymmetric unit for the form *A* crystals and two monomers for the form *B* crystals.

Taken together, the purification of recombinant material produced with the thioredoxin-fusion construct and expressed in *E. coli* Origami strain results in active protein. Nt-CIF shares monomeric behaviour in size-exclusion chromatography (data not shown) with the inhibitor directly purified from tobacco (Weil *et al.*, 1994), but can be produced in higher amounts and purity. We have employed the same expression system to overexpress and purify other invertase inhibitors (unpublished results). The structure determination of Nt-CIF will be published elsewhere. The structure of an invertase inhibitor should yield the first three-dimensional view of a highly regulated physiological system for which only little biochemical information is available so far.

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