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Crystallization and preliminary crystallographic studies of juvenile hormone-binding protein from *Galleria mellonella* haemolymph

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Juvenile hormone-binding protein (JHBP) isolated from *Galleria mellonella* haemolymph has been crystallized using the hanging-drop method in two polymorphic forms. The best diffracting crystals (2.7 Å) are trigonal, space group $P3_121$ (or $P3_221$), with unit-cell parameters a = 110.4, c = 93.9 Å. X-ray diffraction data have been collected for the native crystals using synchrotron radiation and cryogenic conditions (100 K).

1. Introduction

Juvenile hormone (JH) has a profound effect on insects. It regulates embryogenesis (Gilbert *et al.*, 1976), maintains the *status quo* of larva development and stimulates reproductive maturation in the adult forms (Kochman & Wieczorek, 1994). JH is transported from the sites of its synthesis (*corpora allata*; Wiśniewski *et al.*, 1987) to target tissues by a haemolymph carrier called juvenile hormone-binding protein (JHBP). This protects the JH molecules from hydrolysis by non-specific esterases present in the insect haemolymph (Goodman & Chang, 1985).

JHBPs from Lepidoptera are low-molecular-weight (25–35 kDa) monomeric proteins with one specific JH-binding site (de Kort & Granger, 1996; Kochman & Wieczorek, 1994). The JHBP molecule from *Galleria mellonella* consists of 225 amino-acid residues (Rodriguez Parkitna *et al.*, 2002) and does not contain Met or Trp residues (Ożyhar & Kochman, 1987). The protein is glycosylated (Duk *et al.*, 1996) at a position that is as yet unknown. There are four Cys residues per molecule, paired in two disulfide bridges (Kołodziejczyk *et al.*, 2001); however, the topology of the S—S connections has not yet been determined. Binding of JH to *G. mellonella* JHBP induces a profound conformational change in the protein molecule (Krzyżanowska *et al.*, 1998), which may be of importance in the hormone signal transmission (Wieczorek & Kochman, 1991).

Although JHBP proteins have been isolated from several insect species (Kochman & Wieczorek, 1994), present knowledge of JH carriers is limited. Amino-acid sequences of JHBPs from four insect species, including *G. mellonella*, have been reported (Vermunt *et al.*, 2001; Wojtasek & Prestwich, 1995; Touhara *et al.*, 1993; Rodriguez Parkitna *et al.*, 2002). However, there are no experimental data on the three-dimensional structure of a JHBP from any organism. Nevertheless, secondary-structure predictions suggest that JHBP may belong to the calycin superfamily (Rodriguez Parkitna *et al.*, 2002).

In this report, the crystallization conditions and results of preliminary crystallographic analysis of JHBP from *G. mellonella* haemolymph are presented.

2. Materials and methods

2.1. Reagents and chemicals

Ammonium sulfate (AS) was purchased from Roth (Karlsruhe, Germany) and was recrystallized in the presence of 1 m*M* EDTA from water adjusted to pH 7.0 with NH_4OH. Other reagents were of commercially available analytical grade. All solutions were prepared in sodium phosphate buffer, as indicated below.

2.2. Protein purification

Homogeneous JHBP was obtained from *G. mellonella* larvae according to a previously described procedure (Wieczorek *et al.*, 1996). The protein solution was equilibrated against 100 m*M* sodium phosphate buffer pH 7.2 and concentrated to about 1.0 mg ml^{-1} using a Millipore ultrafiltration membrane (PTGC-type, NMWL = 10 000). The JHBP concentration was determined using the extinction coefficient at λ = 280 nm of 0.46 ml mg^{-1} cm^{-1} determined by Krzyżanowska *et al.* (1998).

2.3. Protein crystallization

The stock JHBP solution was dialyzed against or diluted (fourfold) with MilliQ water to a final sodium phosphate concentration of 25 m*M* (pH 7.2) and then concentrated to about 10 mg ml^{-1} using a Centricon 10 Centrifugal Filter Unit (NMWL = 10 000). Crystallization experiments were carried out at 292 K using the hanging-drop vapour-diffusion method (McPherson, 1982). The crystallization drops were mixed using 2 µl of protein solution

Table 1
Dominating forms of JHBP crystals in a two-dimensional screen: pH *versus* ammonium sulfate (AS) concentration (*M*).

Crystallizations were carried out at 292 K. p, precipitate; Ia, trigonal crystals with pentagonal facets; Ib, spindle-shaped trigonal crystals; II, tetragonal bipyramids; ?, unidentified crystal form.

	AS				
pH	1.9	2.1	2.2	2.4	2.6
5.0	?	?	?/II	?	p
6.0	Ia	Ia	II/Ia	Ia	p
7.0	Ia	Ia	Ia/II	Ia	p
8.0	II	II/Ia	II	II/Ia	Ia/Ib
9.0	p	II/Ib	p/Ib	p/Ib	p/Ib

Table 2
Summary of crystal data and data-collection parameters for the trigonal form of JHBP from *G. mellonella* haemolymph.

Values in parentheses are for the highest resolution shell.

Space group	$P3_121$ (or $P3_221$)
Unit-cell parameters (Å, °)	$a = b = 110.4$, $c = 93.9$, $\alpha = \beta = 90$, $\gamma = 120$
No. of observed reflections	120718
No. of unique reflections	18254
Redundancy	6.61 (3.60)
Resolution range (Å)	30.0–2.7 (2.8–2.7)
$\langle I/\sigma(I)\rangle$	24.1 (2.26)
$R_{\rm merge}$	0.061 (0.399)
Mosaicity (°)	0.7
Completeness (%)	98.7 (88.2)

and 2 µl of reservoir solution. The solutions in the reservoirs contained 100 m*M* sodium phosphate buffer and were arranged in the form of a two-dimensional screen (pH values of 3–9 *versus* AS concentrations of 1.9–2.9 *M*; Table 1). Precipitate was observed at pH values below 5 and for AS concentrations above 2.6 *M*. Crystals grew in three morphological forms: polyhedrons with pentagonal facets (form Ia), spindle-shaped (form Ib) and tetragonal bipyramids (form II) (Fig. 1). Form Ia dominates at pH 6–7 and AS concentrations of 1.9–2.4 *M*. At pH 9, form Ib is observed. The small crystals growing at pH 5 are morphologically similar to form Ib crystals obtained at pH 9, but their identity could not be confirmed by diffraction. At pH 8, form II crystals predominate. Some drops contained specimens of two forms (Table 1). Crystals of forms Ia and Ib appeared after about four weeks. The largest crystals of forms Ia and Ib (Figs. 1*a* and 1*b*) reached maximum dimensions of 0.15 × 0.15 × 0.15 and 0.08 × 0.08 × 0.2 mm, respectively, within two months. Form II crystals usually appeared in two weeks. They reached maximum dimensions of 0.1 × 0.1 × 0.2 mm after another four weeks (Fig. 1*c*).

2.4. Diffraction experiments

A complete diffraction data set extending to 2.7 Å resolution was collected from a crystal of form Ia using a 165 mm CCD detector (MAR Research) at beamline I711 of the Lund synchrotron operated at a wavelength λ = 1.095 Å. A summary of the data-collection parameters is presented in Table 2.

The diffraction properties of crystal forms Ib and II were checked using synchrotron radiation at the EMBL BW7B beamline at the DORIS ring of the DESY synchrotron and the I711 beamline of the Lund synchrotron. Because of limited diffraction (up to 5 Å), only a few frames were collected in each case.

In all experiments, the data were collected under cryogenic conditions (100 K) using the flash-freezing method of Teng (1990). Glycerol was mixed with well solutions to a final concentration of 30% and served as cryoprotectant for all forms.

Indexing and integration of the images was performed in *DENZO* and scaling of the intensity data (form Ia only) was performed in *SCALEPACK* from the *HKL* program package (Otwinowski & Minor, 1997).

3. Results and discussion

As shown in Fig. 1, JHBP from *G. mellonella* crystallizes in three morphological forms. The occurrence of these forms is pH-dependent (Table 1). The spindle-shaped crystals (Ib) dominate at pH 9. Those with pentagonal facets (Ia) are present mainly at pH 6–7. The bipyramids (II) were found mainly at pH 8. At pH 5, a number of small crystals of spindle-like shape were present.

The best diffraction could be recorded for the crystals with pentagonal facets (form Ia). It is interesting to note that the pH optimum for these crystals (7.0) is also optimal for JH binding (Ożyhar & Kochman, 1987). Using synchrotron radiation and cryogenic conditions, a 98.7% complete data set characterized by $R_{\rm merge}$ = 0.061 and extending to 2.7 Å resolution could be collected (Table 2). The crystals are trigonal, space group $P3_121$ (or $P3_221$), with unit-cell parameters a = 110.4, c = 93.9 Å, and are most likely to contain three JHBP molecules in the asymmetric unit, corresponding to a Matthews volume (Matthews, 1968) of $V_{\rm M}$ = 2.13 Å^3 Da^{-1} (solvent content 42%). The spindle-shaped crystals (form Ib) are also trigonal, with Bravais lattice and unit-cell parameters identical to those of form Ia. It is therefore reasonable to assume that they represent the same polymorph with a different habit developed under different growth conditions.

The bipyramidal form II crystals have a primitive tetragonal unit cell, with unit-cell parameters a = 98, c = 301 Å. Assuming the 422 class, 3–8 molecules can be expected in the asymmetric unit corresponding to respective $V_{\rm M}$ values of 4.65–1.75 Å^3 Da^{-1} (solvent content 74–30%). The crystals are very fragile, suggesting a high solvent content.

Work on solving the structure of polymorph Ia by the method of isomorphous replacement and on crystallization of the JHBP–JH complex is in progress.

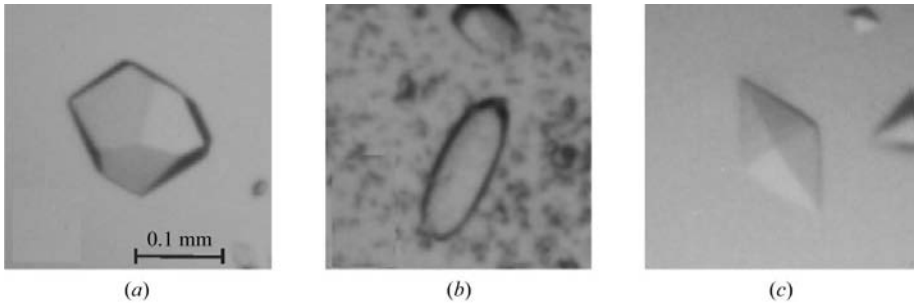

Figure 1
Single crystals of JHBP from *G. mellonella* haemolymph: (*a*) form Ia, (*b*) form Ib, (*c*) form II. The photographs are adjusted to a common scale. All crystals were grown at 292 K using ammonium sulfate as precipitant.

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