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Isolation, purification, crystallization and preliminary X-ray studies of two 30 kDa proteins from silkworm haemolymph

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Juvenile hormone-binding protein (JHBP) and the low-molecular-mass lipoprotein PBMHP-12 belong to a group of 30 kDa proteins that comprise the major protein component of the haemolymph specific to the fifth-instar larvae stage of the mulberry silkworm *Bombyx mori* L. Proteins from this group are often essential for the development of the insect. In a project aimed at crystallographic characterization of *B. mori* JHBP (BmJHBP), it was copurified together with PBMHP-12. Eventually, the two proteins were isolated and crystallized separately. The BmJHBP crystals were orthorhombic (space group $C222_1$) and the PBMHP-12 crystals were triclinic. The crystals diffracted X-rays to 2.9 Å (BmJHBP) and 1.3 Å (PBMHP-12) resolution.

1. Introduction

Major haemolymph proteins, referred to as 30 kDa proteins, are the main protein component of the haemolymph of fifth-instar larvae of the mulberry silkworm *Bombyx mori* L. (Mori *et al.*, 1990). The roles of some of these proteins are still unknown, although many of them are involved in insect development.

A critical role in insect metamorphosis and development is played by juvenile hormone-binding proteins (JHBP), which transport the highly hydrophobic juvenile hormone (JH) from the site of its synthesis (the corpora allata) to target tissues (Goodman & Gilbert, 1974). The presence of the hormone in the haemolymph maintains the larval stage of the insect, whereas a decrease in the JH titre in the haemolymph triggers metamorphosis. The levels of the hormone control the full cycle of insect development from the immature larval stage to the adult form (Riddiford & Truman, 1978). The main role of JHBP is to protect JH and its isoforms (collectively known as JHs) from degradation by esterases (Touhara *et al.*, 1993). There are four classes of JH-binding proteins (Trowell, 1992). The JH-binding proteins studied in this work are highly specific low-molecular-weight proteins that were first described by Trautmann (1972) and Whitmore & Gilbert (1972). To date, structures have been determined for GmJHBP isolated from the haemolymph of the wax moth (*Galleria mellonella*; PDB code 2rck; Kolodziejczyk *et al.*, 2008) and for a recombinant protein from the mulberry silkworm (*Bombyx mori*; PDB code 3a1z; R. Suzuki, A. Fujimoto, T. Shiotsuki, M. Momma, A. Tase, M. Miyazawa & T. Yamazaki, unpublished work) which corresponds to the same coding sequence (gene ID NP_001037074.2; Vermunt *et al.*, 2001) as the BmJHBP protein studied in this work. Structural information obtained from the natural-source protein in our experiments could supplement the previously reported structure, as natural JHBP proteins are usually glycosylated. The expression of the recombinant protein was carried out in *Escherichia coli* (Suzuki *et al.*, 2009) and thus the polypeptide lacks post-translational modifications. Examples of these post-translational modifications have been reported for GmJHBP by Duk *et al.* (1996) and Dębski *et al.* (2004) and documented structurally by Kolodziejczyk *et al.* (2008).

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Table 1
Biophysical parameters for the studied proteins calculated using the *ProtParam* program (http://www.expasy.ch/tools/protparam.html).

Protein	Molecular mass (kDa)	pI	ε_{280} (M^{-1} cm^{-1})
BmJHBP	24.6	4.84	13075
PBMHP-12	28.4	6.37	52892

NMR studies of recombinant JHBP from *B. mori* in complex with JH-III have also been carried out (Suzuki *et al.*, 2009), but it is important to study BmJHBP in its natural form as its post-translational modification may be essential for its proper interaction with hormone molecules. Moreover, crystallographic study of GmJHBP revealed an unexpected second ligand-binding cavity enclosed in the protein fold. The nature of the second ligand is not known. It is more likely that this ligand will be determined in a protein complex obtained from natural material than that obtained from a bacterial cell and again post-translational modification may be necessary for competent ligand binding.

On the other hand, knowledge of the mode of action of JHBPs from several insects may help in the design of novel specific insecticides. For example, *G. mellonella* causes serious damage to beehives (Shimanuki *et al.*, 1992), while the tobacco hornworm *Manduca sexta* and the tobacco budworm *Heliothis virescens* are the most destructive pests of tobacco (Metcalf & Metcalf, 1993). Alignment of the amino-acid sequences of BmJHBP and the JHBP proteins from the above-mentioned insects indicates a high level of homology. The disulfide bridges observed in GmJHBP (Kołodziejczyk *et al.*, 2001) can be predicted to be present in all of the structures, further suggesting a high level of structural similarity. Finally, structural research on haemolymph proteins from *B. mori* is important because this insect is the main producer of silk. Thus, new information about the molecular mechanisms of its development may have commercial potential.

The other protein of interest, PBMHP-12, belongs to the family of low-molecular-weight 30 kDa lipoproteins (Mori *et al.*, 1990) and is involved in the immune response to fungal infection in the haemolymph *via* β-glucan binding (Ujita *et al.*, 2005). β-Glucans may be important virulence determinants since they are the constituents of the cell wall of several pathogenic fungi (Klimpel & Goldman, 1988). By solving the structure of PBMHP-12, we hope to gain insight into the antifungal defence mechanisms used by insects.

2. Materials and methods

2.1. Haemolymph collection

B. mori haemolymph was collected from fifth-instar larvae in aliquots and stored at 198 K. The pooled samples were thawed at 277 K with 0.025 m*M* 1-phenyl-2-thiourea; no protease inhibitors were added. The samples were centrifuged at 18 000*g* for 30 min at 277 K.

2.2. Purification of the proteins

The purification procedure led to the isolation of two proteins from mulberry silkworm haemolymph: BmJHBP and PBMHP-12. The first step consisted of gel filtration, which was carried out using a Superdex 200 pg column (XK 16/100, Amersham Biosciences) equilibrated with 100 m*M* NaCl, 10 m*M* Tris pH 7.3 and 0.025 m*M* 1-phenyl-2-thiourea. The collected fractions containing 30 kDa proteins were concentrated and applied onto a Q Sepharose column (XK 16/10, Amersham Biosciences) equilibrated with 30 m*M* NaCl, 5 m*M* Tris pH 7.3. Stepwise elution was chosen as a method for protein separation in ion-exchange chromatography. PBMHP-12 was eluted with 60 m*M* NaCl and BmJHBP was eluted with 90 m*M* NaCl. The collected peak fractions of both proteins were concentrated to 10 mg ml^{-1}. The

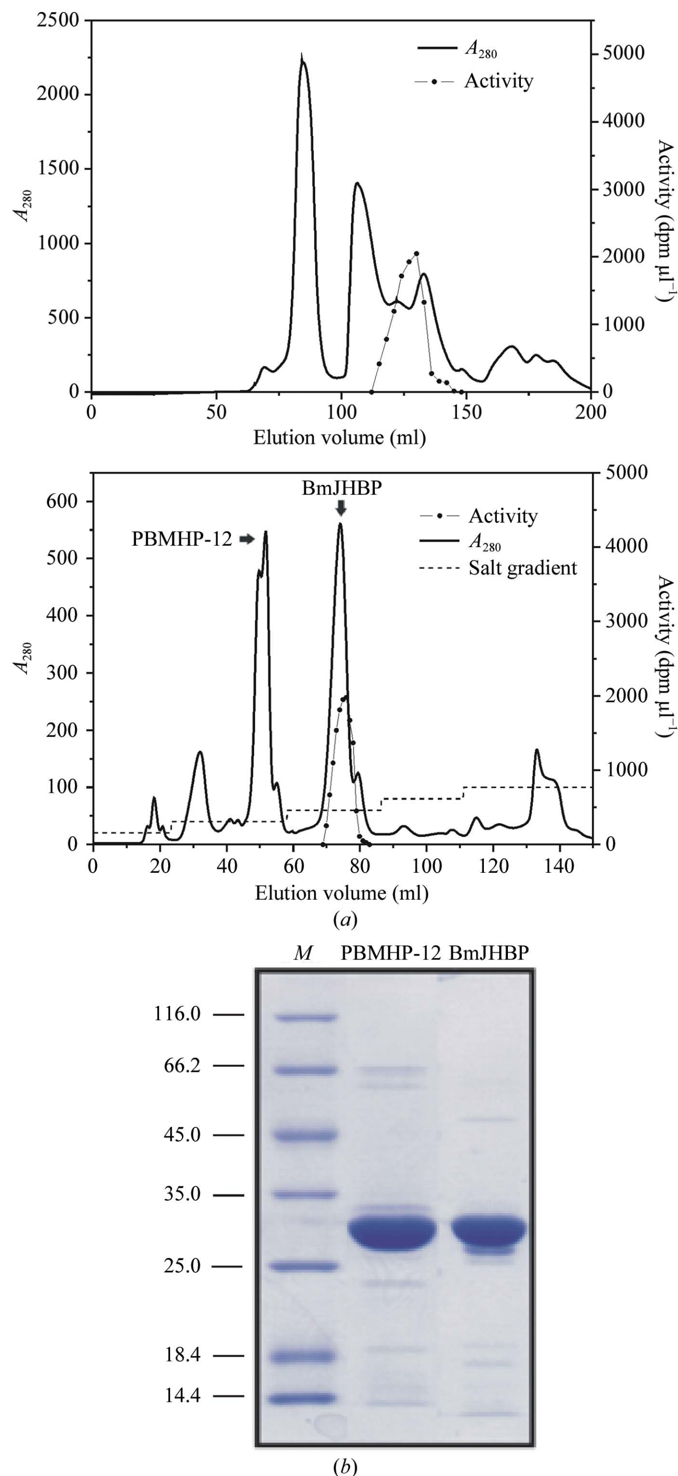


Figure 1
Purification of the proteins. (*a*) Elution peaks from two FPLC purification steps: gel-filtration (top) and ion-exchange chromatography (bottom). The activity curves for BmJHBP correspond to a JH-binding assay (Ożyhar & Kochman, 1987); they indicate the presence of radioactive-labelled JH molecules bound by BmJHBP in the samples. (*b*) SDS–PAGE electrophoregrams of the peak fractions of PBMHP-12 and BmJHBP from the last purification step (lane *M* contains Protein Molecular Weight Marker from Fermentas); these protein samples were used in the crystallization experiments.

protein concentration was determined by measuring the absorption at 280 nm (using the theoretical extinction coefficient; see Table 1) and/or by using the Bradford assay (Bradford, 1976). The yield of purification for both proteins was approximately 2 mg protein from 3 ml haemolymph. A JH-binding assay (Ożyhar & Kochman, 1987) was used to detect BmJHBP in the collected fractions, as shown in Fig. 1(*a*). The second protein was identified as PBMHP-12 by sequence analysis using liquid chromatography and electrospray ionization tandem mass spectrometry (LC/MS/MS; McCormack *et al.*, 1997).

Moreover, MALDI–TOF MS analysis was carried out to determine the molecular mass of both proteins (Fig. 2). The theoretical molecular mass, pI and extinction coefficients (Table 1) were calculated with *ProtParam* (http://www.expasy.ch/tools/protparam.html).

2.3. Crystallization

The proteins were crystallized in 24-well hanging-drop plates using the vapour-diffusion method. The reservoir volume was 0.5 ml and the crystallization drops were made up of 1 µl protein solution and 1 µl reservoir solution. The initial crystallization conditions were found using commercially available screening solutions (PEG/Ion and Index, Hampton Research).

PBMHP-12 crystallized in 0.1 *M* HEPES buffer pH 7.5, 22% PEG 3350 and a KSCN concentration range from 100 to 250 m*M*. Single crystals (Fig. 3*a*) grew to dimensions of 0.35 × 0.20 × 0.10 mm using 200 m*M* KSCN.

The crystals of BmJHBP were obtained using 0.1 *M* bis-Tris buffer pH 6.5, 30% PEG MME 550 and 0.1 *M* $CaCl_2$. To improve the crystal morphology, various additives were tried (Additive Screen, Hampton Research). The best results were obtained using NDSB (Fig. 3*b*), sarcosine and KCl.

2.4. Data collection

X-ray diffraction data were collected on beamline 14.1 of the BESSY synchrotron, Berlin, Germany. The detector was a MAR 225 CCD and data were recorded from single crystals using the rotation method with an oscillation of 0.5° at a temperature of 100 K. A solution consisting of 0.1 *M* HEPES pH 7.0, 50% PEG 400 and 200 m*M* KSCN mixed with the reservoir solution (in a 1:1 ratio) was used as a cryoprotectant for PBMHP-12. Before cryocooling, the crystals were transferred into the cryoprotectant solution for a few seconds. No cryoprotection was used for BmJHBP because PEG MME 550 was present in the reservoir solution. All crystals were cryocooled in a stream of nitrogen gas. The collected images were indexed, integrated and scaled using *XDS* (Kabsch, 2010).

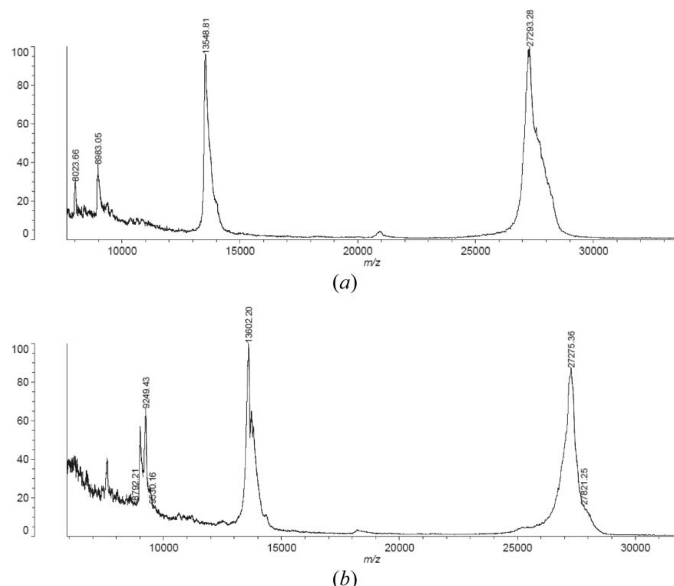
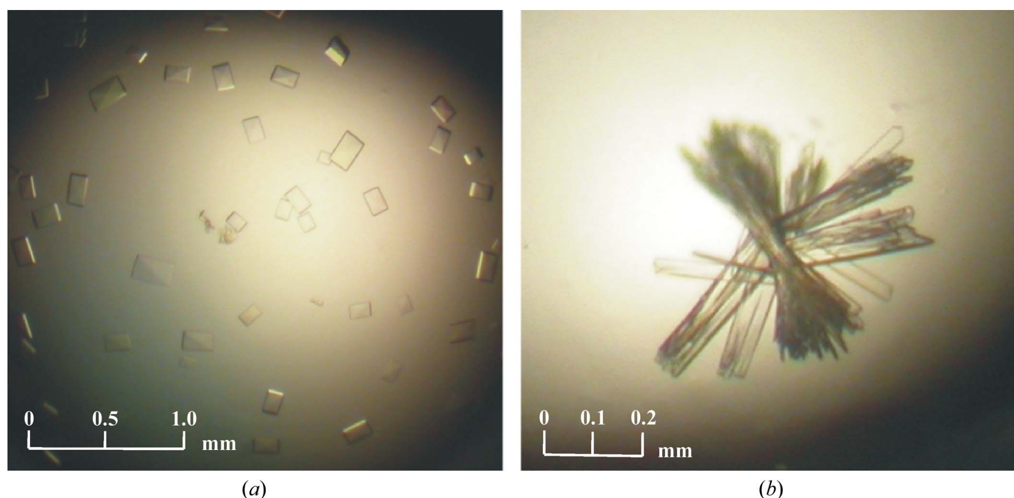


Figure 2
MALDI–TOF MS spectra for (*a*) PBMHP-12 and (*b*) BmJHBP.

3. Results and discussion

Two major haemolymph proteins from silkworm (*B. mori*) were isolated, purified and crystallized for structural studies. The yield of the purification method was quite high (approximately 0.6 mg ml^{-1} in both cases). The amount and the purity of the proteins were sufficient for successful crystallization.

MS-based sequence analysis indicated that the unknown protein was PBMHP-12. Nine peptides were identified and found to perfectly match the corresponding PBMHP-12 sequence with a coverage of 40.15%, which sets the level of confidence in the identification of this protein at 89%. Other top-ranking proteins (PBMHPC-23 and PBMHPC-19) also belong to the 30 kDa lipoprotein family, but the



(*a*) (*b*)

Figure 3
Crystals of the studied 30 kDa haemolymph proteins from *B. mori*. (*a*) Single crystals of PBMHP-12. (*b*) Clusters of BmJHBP crystals.

Table 2
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

	PBMHP-12	BmJHBP
No. of crystals	1	1
Space group	*P*1	$C222_1$
Unit-cell parameters (Å, °)	a = 42.4, b = 50.1, c = 55.3, α = 93.4, β = 94.7, γ = 102.7	a = 56.4, b = 117.0, c = 116.0, $\alpha = \beta = \gamma$ = 90.0
Molecules per asymmetric unit	2	1 or 2
V_M (Å^3 Da^{-1})	2.10	3.92 or 1.96
Solvent content (%)	41.5	68.6 or 37.2
Temperature (K)	100	
Radiation source	Synchrotron [BESSY beamline 14.1]	
Wavelength (Å)	0.918	
Detector	MAR CCD 225 mm	
Crystal-to-detector distance (mm)	140	289
Rotation range per image (°)	0.5	0.5
Total rotation range (°)	360	360
Exposure time per image (s)	5.2	8.0
Resolution (Å)	50–1.3 (1.4–1.3)	50–2.9 (3.0–2.9)
Mosaicity† (°)	0.44	0.71
Total no. of measured intensities	372101	116975
Unique reflections	97046	8793
R_{merge} (%)	6.3 (30.9)	13.3 (54.2)
R_{meas} (%)	7.3 (36.0)	13.8 (56.6)
Multiplicity	3.8 (3.7)	13.3 (11.2)
$\langle I/\sigma(I) \rangle$	13.7 (4.64)	17.5 (5.65)
Completeness (%)	89.3 (63.8)	99.5 (96.0)
Wilson *B* factor (Å^2)	16.3	41.7

† As calculated by *XDS* (Kabsch, 2010).

values for the sequence coverage and the level of confidence were significantly lower. The correctness of this analysis will be confirmed by structure determination.

The molecular mass calculated for BmJHBP according to the amino-acid sequence (Table 1) is slightly lower than that determined by MALDI–TOF MS (Fig. 2), suggesting post-translational modifications of some residues. The additional 3 kDa of mass could correspond to sugar residues, which could be attached to the protein at the two potential glycosylation sites. In contrast to the case of BmJHBP, the molecular weight of PBMHP-12 determined by MALDI–TOF MS analysis is smaller by about 1 kDa than the theoretical mass. The reason could be partial degradation of the protein during purification or, alternatively, the initial signal peptide could be longer than predicted previously (Sakai *et al.*, 1988).

The crystallization conditions which yielded BmJHBP crystals are promising for further experiments aimed at obtaining BmJHBP–JH complexes. The main precipitant is PEG MME 550, in contrast to the GmJHBP crystallization conditions, in which ammonium sulfate was used (Kołodziejczyk *et al.*, 2003) and was found to damage crystals of GmJHBP–JH complexes (Kolodziejczyk *et al.*, 2008). The crystallization conditions also differ from the conditions that were used for the recombinant protein, in which the main precipitant was 2-methyl-2,4-pentanediol. The pH of 6.5 for the reservoir solution in our experiment was adjusted using bis-Tris buffer, in contrast to the case of the recombinant protein, which was characterized by pH 7.0 and the use of cacodylate buffer. Our conditions are closer to those found in the haemolymph, in which the pH is about 6.5 (Wyatt *et al.*, 1956).

About half a dozen BmJHBP crystals were examined by X-ray diffraction; all of them possessed the same unit-cell parameters and belonged to space group $C222_1$. Their diffraction properties were not good, with a resolution of about 3.5–2.9 Å. The data-collection statistics for the best crystal are shown in Table 2. Using the MS-determined molecular mass, Matthews coefficients (Matthews, 1968) were calculated for two possible contents of the asymmetric unit. The presence of one protein molecule in the asymmetric unit yielded a V_M value of 3.92 Å^3 Da^{-1} (solvent content 68.6%) and the presence of two molecules in the asymmetric unit yielded a value of 1.96 Å^3 Da^{-1} (solvent content 37.2%). A Patterson self-rotation function did not show any noncrystallographic axial symmetry, so it is reasonable to assume that there is a single copy of the protein in the asymmetric unit. The crystals of the recombinant protein, which yielded the 3a1z structure deposited in the PDB (R. Suzuki, A. Fujimoto, T. Shiotsuki, M. Momma, A. Tase, M. Miyazawa & T. Yamazaki, unpublished work), belonged to space group $P2_12_12_1$, with unit-cell parameters a = 54.9, b = 114.7, c = 192.9 Å, *i.e.* they are different from those determined by us for BmJHBP isolated directly from the insect haemolymph (space group $C222_1$, a = 56.4, b = 117.0, c = 116.0 Å; Table 2).

Attempts to solve the BmJHBP structure by molecular replacement have so far been unsuccessful. The statistics of *E*-value distribution calculated in *XDS* indicated a twinning problem. However, since no merohedral twinning is possible in this space group, the character of the twinning is not obvious. Further crystallization experiments are under way with the aim of growing crystals that are not affected by twinning. In order to obtain untwinned crystals with better diffracting properties, an alternative purification strategy has been considered. A promising alternative is the inclusion of hydrophobic interaction chromatography as the last purification step.

The crystals of PBMHP-12 were triclinic and diffracted X-rays to 1.3 Å resolution. The asymmetric unit was estimated to contain two protein molecules, with a Matthews coefficient of 2.10 Å^3 Da^{-1} (solvent content 41.5%). Detailed data statistics are presented in Table 2. There is no suitable model for use in molecular-replacement calculations. Thus, other methods based on heavy-atom derivatives for solution of the phase problem are currently being considered. The crystals were of good quality and their derivatives with halides and heavy metals such as Hg or Pt were sufficiently stable for diffraction experiments. Initial diffraction data for derivative crystals showed insufficient anomalous signal; therefore, optimization of the derivatization procedures is currently under way.

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