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Optimization of crystals from nanodrops: crystallization and preliminary crystallographic study of a pheromone-binding protein from the honeybee *Apis mellifera* L.

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Pheromone-binding proteins (PBPs) are small helical proteins (~13–17 kDa) present in various sensory organs from moths and other insect species. They are involved in the transport of pheromones from the sensillar lymph to the olfactory receptors. Here, crystals of a PBP (Amel-ASP1) originating from honeybee (*Apis mellifera* L.) antennae and expressed as recombinant protein using the yeast *Pichia pastoris* are reported. Crystals of Amel-ASP1 have been obtained by the sitting-drop vapour-diffusion method using a nanodrop-dispensing robot under the following conditions: 200 nl of 40 mg ml^{-1} protein solution in 10 m*M* Tris, 25 m*M* NaCl pH 8.0 was mixed with 100 nl of well solution containing 0.15 *M* sodium citrate, 1.5 *M* ammonium sulfate pH 5.5. The protein crystallizes in space group $C222_1$, with unit-cell parameters a = 74.8, b = 85.8, c = 50.2 Å. With one molecule in the asymmetric unit, V_M is 3.05 Å^3 Da^{-1} and the solvent content is 60%. A complete data set has been collected at 1.6 Å resolution on beamline ID14-2 (ESRF, Grenoble). The nanodrop crystallization technique used with a novel optimization procedure made it possible to consume small amounts of protein and to obtain a unique crystal per nanodrop, suitable directly for data collection in-house or at a synchrotron-radiation source.

1. Introduction

Honeybees, like most insects, use small signalling chemical compounds to recognize and respond to their environment and to congeneric animals. In insects, odorant-binding proteins (OBPs) are small helical proteins which ferry these compounds from air to the olfactory receptors through the sensillar lymph (Vogt & Riddiford, 1981; Vogt *et al.*, 1991). Pheromone-binding proteins (PBPs) are a subclass of OBPs which carry pheromonal molecules to their receptors, which then induces sexual or endocrine responses to cognates (Krieger *et al.*, 1996).

In the honeybee antennae, three different classes of antennal-specific proteins (ASPs) have been identified as OBPs, namely ASP1, ASP2 and ASP3 (Danty *et al.*, 1998). Whereas ASP2 was assigned to be a general odorant-binding protein (Briand *et al.*, 2001) and ASP3 classified as a chemosensory protein (Briand *et al.*, 2002), ASP1 (Amel-ASP1) was shown to be associated with queen-pheromone detection and to be able to bind 9-keto-2(*E*)-decenoic acid and 9-hydroxy-2(*E*)-decenoic acid, the most active components of the queen-pheromone blend (Danty *et al.*, 1999). ASP1 should therefore be considered as a hymenopteran pheromone-binding protein. To date, only the three-dimensional structure of a lepidopteran PBP originating from *Bombyx mori* has been solved, both free and in complex with its pheromone, the alkyl unsaturated alcohol bombykol (Sandler *et al.*, 2000; Horst *et al.*, 2001; Lee *et al.*, 2002). This crystallographic structure revealed a new α-helical fold delineating a buried cavity filled with bombykol. However, its sequence homology with Amel-ASP1 is too low (15% identity) to allow reasonable three-dimensional structure prediction (Fig. 1). Here, we report the

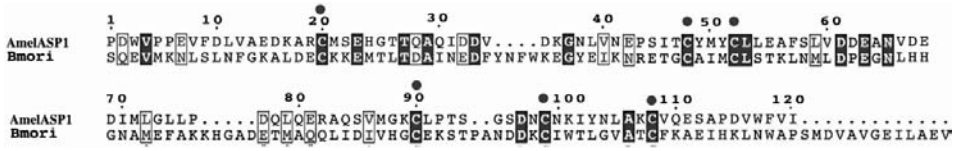

Figure 1
Alignment of the sequence of Amel-ASP1 with that of the PBP from *Bombyx mori* (Bmori) of known three-dimensional structure. Conserved residues are identified with a black background. Homologous residues are boxed. The six conserved cysteines are identified with circles.

crystallization of Amel-ASP1, a dimer of $2 \times 13\,180$ Da. Crystals were obtained using the nanodrop crystallization technique, first developed in response to the high demand of structural genomics programmes, followed by a novel optimization procedure (Sulzenbacher *et al.*, 2002). The crystals diffract to 1.6 Å at synchrotron sources, are suitable for high-resolution structure determination and are amenable for solution with MAD methods (Hendrickson, 1991) at the Se edge after labelling using the procedure of Larsson *et al.* (2002) with *Pichia pastoris*.

2. Material and methods

2.1. Crystallization screening

Cloning, expression and purification of Amel-ASP1 have been described elsewhere (Danty *et al.*, 1999). The protein expressed using *P. pastoris* was concentrated (Nanosep-3, Filtron) and washed with 10 m*M* Tris–HCl, 25 m*M* NaCl pH 8.0 to a concentration of 40 mg ml^{-1} as determined by spectrophotometry using the theoretical ε_{280} of 15 580 M^{-1} cm^{-1}.

Screening experiments were performed with several commercial kits (for details, see Sulzenbacher *et al.*, 2002). The nanodrop crystallization experiments were performed using the sitting-drop method in Greiner plates (Mueller *et al.*, 2001). The reservoirs of the Greiner plates were filled using a TECAN pipetting robot, while the nanodrops were dispensed by a Cartesian Inc. robot (Sulzenbacher *et al.*, 2002) contained in a closed cabinet with a controlled humidity level to avoid evaporation of the drops. Small volumes of 40 mg ml^{-1} protein sample were aspirated by eight tips in parallel; for each well of the 8×12 Greiner plates, volumes of 200 nl were dispensed onto the central of three sitting-drop shelves. Subsequently, 100 nl of the reservoir solutions were aspirated by the eight tips and added to the protein drops. This operation was repeated 12 times until the eight rows were complete. Finally, the plates were sealed with a transparent film and stored in a cabinet at 223 K.

2.2. Crystal optimization

The crystallization plates containing the first screening experiments were regularly observed using a Nikon microscope equipped with a video camera and an *XY* computer-driven plate holder. Optimization of crystallization conditions was carried out employing solutions from an optimization matrix, dispensed by robots, by varying two parameters at a time (pH and precipitant concentration; Fig. 2*a*), exploring the parameter space around the initial conditions identified during the screening step. Matrices of 8×8 conditions were established and varying amounts of four stock solutions were distributed and subsequently mixed by the TECAN robot within the 64 wells of the Greiner plates (Fig. 2*a*). These plates were subsequently used for nanodrop crystallization using the Cartesian Inc. robot dispensing 100 nl of precipitant over 200 nl of protein solution at 40 mg ml^{-1}.

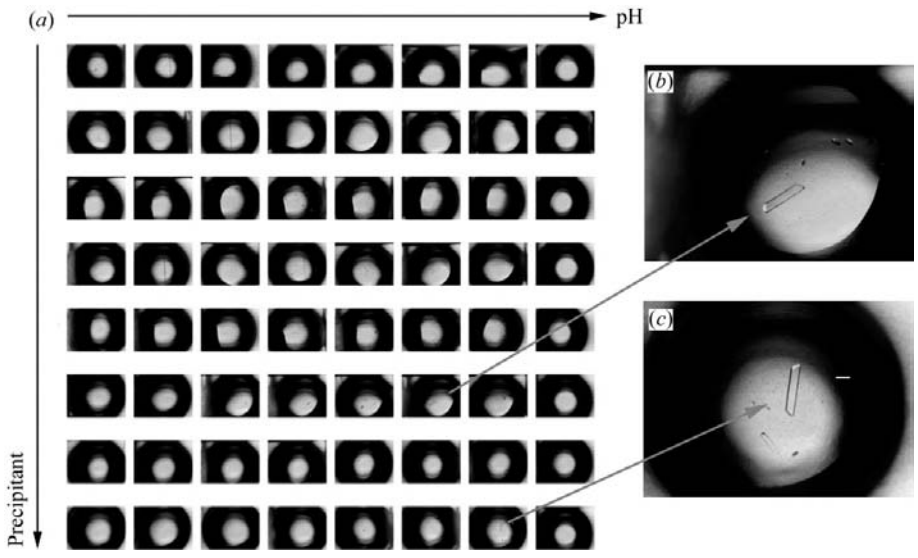

Figure 2
Crystal optimization of Amel-ASP1. (*a*) The pH range is 5.0–6.0 and the precipitant (ammonium sulfate) concentration range is 1.3–1.7 *M*. (*b*) and (*c*) Two drops in the lower right corner yielded unique crystals useable for data collection of approximate dimensions $40 \times 80 \times 400$ µm. The scale bar in (*c*) represents 100 µm.

3. Results and discussion

3.1. Crystallization of Amel-ASP1

The initial crystallization droplet contained 200 nl of 40 mg ml^{-1} protein solution in 10 m*M* Tris, 25 m*M* NaCl pH 8.0 mixed with 100 nl of well solution containing 1.5 *M* ammonium sulfate, 0.15 *M* sodium citrate pH 5.5. Initial thin crystals of dimensions $\sim 0.02 \times 0.1 \times 0.5$ mm appeared after one to three weeks. Thicker crystals ($0.2 \times 0.1 \times 0.7$ mm) were obtained after optimization (Figs. 2*b* and 2*c*). Crystals have also been obtained at 293 K using the classical hanging-drop vapour-diffusion method in Linbro boxes. The initial droplet contained a volume of 1000 nl of a 40 mg ml^{-1} protein solution in 10 m*M* Tris, 25 m*M* NaCl pH 8.0 mixed with 1000 nl of well solution containing 1.6 *M* ammonium sulfate, 0.15 *M* sodium citrate pH 5.5. Crystals of dimensions $0.1 \times 0.1 \times 0.3$ mm appeared after one to three weeks.

Crystal improvement with nanodrops was particularly important because protein at high concentration was required for crystallization and only a few milligrams were available to carry out the crystallization. The required protein quantity for the screening experiment was less than 3 mg and another 0.5 mg were necessary for optimization.

3.2. Diffraction and data collection

Diffraction data were obtained on a rotating-anode generator or at a synchrotron-radiation source. Crystals were cryocooled at 100 K in their crystallization liquor with the addition of 25% glycerol as a cryoprotectant. Diffraction images were indexed and integrated with *DENZO* (Otwinowski & Minor, 1997) and scaled with *SCALA* (Collaborative Computational Project, Number 4, 1994). Amel-ASP1 crystallizes in space group $C222_1$, with unit-cell parameters $a = 74.8$, $b = 85.8$, $c = 50.2$ Å. With one molecule in the asymmetric unit, V_M is 3.05 Å^3 Da^{-1}, corresponding to a solvent content of 60% (Matthews, 1968).

Data were first collected in-house with a MAR-345dtb detector on a Rigaku RU-200B source using a crystal originating from optimized nanodrops. Diffraction extended to 2.9 Å resolution with an $R_{\rm merge}$ of 8.4% (Table 1). Crystals obtained in Linbro boxes or from optimized nanodrops were collected at beamline ID14-2 at ESRF (Grenoble), tuned to 0.933 Å, with an ADSC-Q4 detector. A full diffraction data set was collected with the latter crystal at 1.6 Å resolution using 110 1° oscillation images with 12 s exposure time. The diffraction data

Table 1
Data-reduction statistics.

Values in parentheses are for the last resolution shell.

Initial drop size	300 nl	2 µl	300 nl
X-ray source	Rotating anode	ID14-EH1	ID14-EH2
λ (Å)	1.5418	0.933	0.933
Resolution range (Å)	30.0–2.9 (3.06–2.90)	28.4–2.0 (2.11–2.0)	28.4–1.6 (1.66–1.6)
R_{sym} (%)	8.4 (26.7)	3.9 (9.5)	3.6 (26.7)
$I/\sigma(I)$	5.0 (2.4)	7.2 (3.1)	10.2 (2.6)
Completeness	99.8 (99.8)	99.1 (99.1)	99.7 (99.7)
Multiplicity	4.9 (5.0)	5.4 (5.5)	3.8 (3.8)

have an R_{merge} of 3.2% and a completeness of 99.7%. Data-collection statistics are summarized in Table 1.

4. Conclusion

Nanodrop crystallization screening followed by automated optimization proved to be an efficient method for obtaining diffracting crystals with small amounts of protein. Amel-ASP1 crystals obtained from nanodrop crystallization diffracted to average resolution on a rotating source or to high resolution at synchrotron-radiation sources. The crystal obtained with classical microdrop techniques diffracted to lower resolution than the crystal obtained in nanodrops after optimization (Table 1). This indicates that crystals obtained with the nanodrop crystallization technique can provide comparable or better diffraction than those obtained with classical methods.

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