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CRYSTALLIZATION ANNOUNCEMENTS

Crystallization of Glycosylated and Nonglycosylated Phytohemagglutinin-L

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ABSTRACT **In the seeds of legume plants a class of sugar-binding proteins can be found, generally called legume lectins. In this paper we present the crystallization of phytohemagglutinin-L (PHA-L), a glycosylated lectin from the seeds of the common bean (*Phaseolus vulgaris*). Single PHA-L crystals were grown by vapor diffusion, using PEG as precipitant. The protein crystallizes in the monoclinic space group *C*2, and diffracts to a resolution of 2.7 Å. The unit cell parameters are $a = 106.3$ Å, $b = 121.2$ Å, $c = 90.8$ Å, and $\beta = 93.7°$. The asymmetric unit probably contains one PHA-L tetramer. Crystals of a recombinant nonglycosylated form of PHA-L, grown under identical conditions, and crystals of the native PHA-L, grown in the presence of isopropanol, did not survive the mounting process.**

Key words: lectins, crystallization, X-ray diffraction

INTRODUCTION

Lectins are proteins of nonimmune origin that bind complex carbohydrates specifically and reversibly, often with hemagglutinating properties. They are very abundant in nature, and can be found in a wide variety of plants and animals. Structural biologists are using lectins as a model to understand the general principles and mechanisms of protein–carbohydrate interactions. The most extensively studied lectins are the lectins found in the seeds of the legume plants.[1] Their physiological function in the plant still remains unsure, but involvement in defense against predation[2] and interaction with *Rhizobium* symbionts have been suggested.[3] Despite the highly conserved tertiary structure of these lectins, their quaternary structures and sugar specificities differ considerably. The currently solved structures reveal that the monomers can associate into a number of different types of tetramers (concanavalin A,[4] peanut lectin[5]) and dimers (lentil lectin,[6] lectin IV from *Griffonia simplicifolia*,[7] *Erythrina corallodendron* lectin[8]). In the seeds of the common bean (*Phaseolus vulgaris*) a protein fraction can be found with hemagglutinating and sugar binding properties, called phytohemagglutinin (PHA). It consists of five different tetrameric glycoproteins, composed of two different types of subunits associated in all possible combinations. These subunits are the leucoagglutinating L-type subunit (252 AA) and the erythroagglutinating E-type subunit (254 AA),[9] and the five possible tetramers formed are E_4, E_3L, E_2L_2, E_1L_3, and L_4. The L-type and E-type subunits are both members of a family of four different polypeptides encoded by four tightly linked genes, generally referred to as the phytohemagglutinin family of bean proteins. This family further consists of arcelin[10] (a protein with entomotoxic properties) and an α-amylase inhibitor[11] (inhibiting α-amylases of animal origin). Phytohemagglutinin-L (PHA-L) consists solely of four L-type subunits. Each subunit is N-glycosylated at two different sites, with consensus sequence Asn-X-Ser/Thr. It possesses a high-mannose type sugar attached at Asn-12, and a complex type sugar at Asn-60.[12] The minimal structural unit for high-affinity binding is the pentasaccharide Galβ1→4GlcNAcβ1→2(Galβ1→4GlcNAcβ1→6)Man, which is found in tetra- and triantennary complex type oligosaccharides.[13] Because of the notorious difficulties in crystallizing glycoproteins, we decided to

Abbreviations used: PEG, polyethylene glycol; PHA-L, phytohemagglutinin-L; PBS, phosphate-buffered saline.

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crystallize both native (glycosylated) and recombinant (nonglycosylated) PHA-L.

MATERIALS AND METHODS

Protein Expression and Purification

For the expression of PHA-L in maturing canola (*Brassica napis*) seeds, we excised the chimerical phaseolin/PHA-L gene from binary vector constructs, previously used for transforming tobacco with a mutant of PHA-L that has two mutated sugar attachment sites,[14] and introduced it into pBluescript (Stratagene). This allowed the addition of the *Bam*HI site at the 3′ end of the gene. Finally we inserted the gene as a *Hin*dIII/*Bam*HI fragment into the binary vector pCGN1578,[15] resulting in pCGN3832. This construct was used to transform the *Brassica napus* cultivar 211/8,[16] and transgenic plants were regenerated. These plants expressed high levels of nonglycosylated PHA-L in their seeds. The seeds were ground and the resulting fine powder was suspended in 20 ml of PBS per g powder. The suspension was stirred overnight at 4°C. Since the seeds contain a considerable amount of oil, the suspension was defatted by centrifuging three times at 12,000*g* for 15 min at 4°C, and filtering the supernatant over a piece of cloth. The recombinant PHA-L was isolated from this extract by affinity adsorption on porcine thyroglobulin-sepharose, as described by Felsted et al.[17] The protein was transferred from the elution buffer to PBS with the aid of a PD-10 column (Sephadex G-25M, Pharmacia). Further concentration of the purified recombinant PHA-L was accomplished by ultrafiltration in an Amicon cell (Millipore filter, MW cut-off 10 kDa). The concentration of the protein was determined by measuring the absorption at 280 nm, assuming an absorption of 1.0 for a protein concentration of 1.0 mg/ml and a 1.0 cm path length. Starting from 50 g of ground seeds, the purification yielded 50 mg recombinant, nonglycosylated PHA-L. The solution was stored at −20°C at a protein concentration of 5 mg/ml.

Native (glycosylated) PHA-L was purchased from Sigma. The lyophilized powder was dissolved in distilled deionized water to a suitable concentration for the crystallization trials. The purity of the samples was judged by gel electrophoresis in 15% polyacrylamide running gels. This yielded two diffuse bands very close together around 35 kDa for the native PHA-L solution. The recombinant, nonglycosylated PHA-L solution showed one major band around 26 kDa, and a very faint minor band just below the first.

Crystallization

Suitable crystallization conditions for both the glycosylated and the nonglycosylated recombinant PHA-L were screened by vapor diffusion using the hanging drop method. The droplets were prepared on silanized coverslips, by mixing 5 μl protein solution and 5 μl bottom solution. The best crystals for both the native and the recombinant PHA-L were obtained with a bottom solution containing 8% (w/v) PEG 6 kDa (Janssen Pharmaceutical) and 100 mM Tris, brought to pH 8.5 with HCl, and a protein solution of 5 mg/ml. These crystals were obtained after 1 week of incubation at 4°C or at room temperature. The latter showed visible signs of degradation after 3 weeks, while the former remained stable in the mother liquor. For the native PHA-L, a second set of conditions, which yielded crystals with a different habit, was found. In this case, the protein concentration was 10 mg/ml. Bottom solutions containing isopropanol in a 20 to 30% range (v/v) and 100 mM Na-citrate, brought to pH 5.6 with citric acid, yielded crystals after an incubation of 1 to 3 weeks at 4°C.

X-Ray Diffraction Study

Crystals of native PHA-L, grown at room temperature in the presence of PEG, were transferred from a hanging drop into a 0.5-mm glass capillary tube. Diffraction analysis was performed on an Enraff-Nonius FAST area detector, using CuK_α radiation generated by a rotating anode X-ray generator (40 kV, 98 mA), and filtered by a Ni filter. The crystal to detector distance was 120 mm. Autoindexing was done using the MADNESS software packet.[18] The recombinant PHA-L crystals, and the native PHA-L crystals grown in the presence of isopropanol, did not survive the mounting process. During the mounting of the native PHA-L crystals grown in the presence of isopropanol, evaporation of the mother liquor and precipitation on the crystals occurred, rendering them useless for further work. The recombinant PHA-L crystals cracked upon withdrawal of the mother liquor.

RESULTS AND DISCUSSION

The crystals of native, glycosylated PHA-L (Fig. 1A), grown in the presence of PEG at room temperature, could be used to record a useful X-ray diffraction data set. These crystals were monoclinic, space group $C2$, with cell parameters $a = 106.3$ Å, $b = 121.2$ Å, $c = 90.8$ Å, $\beta = 93.7°$. The unit cell volume is consistent with one PHA-L tetramer in the asymmetric unit, corresponding to a crystal volume per protein mass (V_m) of 2.1 Å^3/Da and a solvent content of approximately 42.4%. This falls well within the range of the solvent content of other protein crystals.[19] A total of 67,416 intensities between 14.96 and 2.70 Å was reduced to 27,844 unique reflections, with a merging R-factor of 0.10. The statistics of the data collection are given in Table I.

Crystallization of extensively glycosylated proteins faces considerable difficulties. Heterogeneity of glycosylation and the inherent flexibility of the carbohydrate moieties, which are present on the sur-

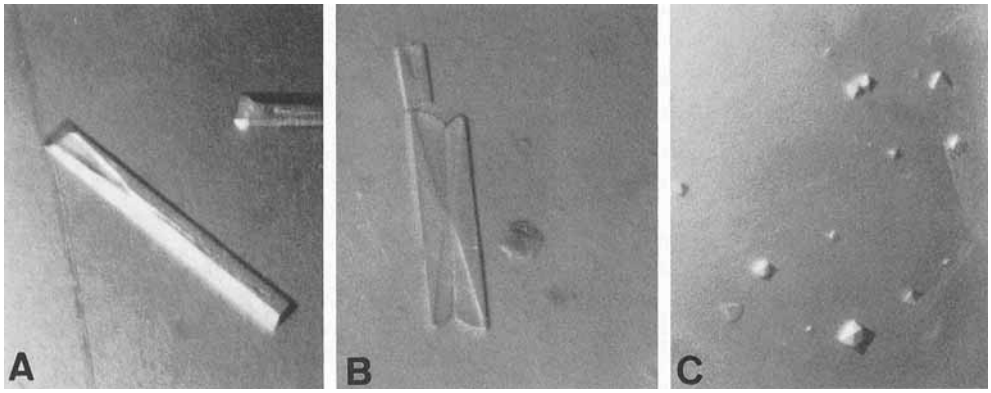

Fig. 1. Microphotographs of typical crystals from (**A**) the native PHA-L grown in the presence of PEG 6K, (**B**) the nonglycosylated recombinant PHA-L grown in the presence of PEG 6K, and (**C**) the native PHA-L grown in the presence of isopropanol. See the text for details.

TABLE I. Data Collection Statistics of the Native PHA Crystal

Resolution (Å)	Number of observed reflections	Number of unique reflections	Completeness (%)	R_{merge}*
14.98–7.51	2149	1246	95.9	0.040
7.51–5.68	4257	1864	96.6	0.078
5.68–4.75	8401	2317	96.9	0.080
4.75–4.17	9795	2700	97.4	0.088
4.17–3.76	8016	2929	94.2	0.097
3.76–3.45	6830	3102	90.9	0.085
3.45–3.20	6965	3287	89.2	0.124
3.20–3.01	7005	3428	87.0	0.177
3.01–2.84	7014	3468	83.0	0.262
2.84–2.70	6984	3503	79.5	0.355
Total	67416	27844	88.9	0.099

*$R_{merge} = \Sigma_i\Sigma_j(I_{ij} - \langle I_j \rangle)/\Sigma_i I_{ij}$.

face of the protein, often impair the formation of a sufficiently homogeneous crystal. PHA-L has about 10% w/w attached carbohydrate. Therefore we decided to try to crystallize a recombinant, nonglycosylated form of PHA-L as well as the native form. Surprisingly, it was possible to obtain a sufficient number of good quality crystals from the native, glycosylated PHA-L, while the crystals from the recombinant, nonglycosylated form (Fig. 1B), grown under identical conditions, turned out to be very fragile. The latter cracked upon withdrawal of the mother liquor during the mounting process and could subsequently not be used to record a data set. Damage during the mounting process also occurred with the native PHA-L crystals grown in the presence of isopropanol as a precipitant (Fig. 1C). These crystals show a habit that differs from the habit of the native PHA-L crystals obtained in the presence of PEG.

A possible explanation of the difference in quality between the crystals of the native and the recombinant PHA-L is that the two covalently bound sugars per monomer influence the packing of the subunits, as is the case with the *Erythrina corallodendron* lectin[7] and lectin IV from *Griffonia simplicifolia*.[6] In the latter two cases the presence of N-linked oligosaccharides is thought to impair the formation of the standard dimer, and to cause the lectin to adopt a different quaternary structure. Hence, it will be very interesting to verify the influence of the glycosylation on the quaternary structure, and to compare the structure of PHA-L with the other solved legume lectin structures. At present the structures of only two legume lectin tetramers have been solved with X-ray diffraction, namely concanavalin A[3] and peanut lectin.[4] The packing of the subunits in these tetramers differs considerably, despite the high structural homology of the monomers. The legume lectins are thus an ideal model system to study the rules behind quaternary organization. Possibly a solved structure of PHA-L will reveal yet a third way for the legume lectin monomers to associate into tetramers. This solved structure would then prove to be a valuable addition to the set of solved legume lectin structures. We are currently attempting to solve the structure of the native, glycosylated PHA-L. In addition, we are also attempt-

ing to grow crystals of native PHA-L complexed with various oligosaccharides.

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