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Expression of Winged Bean Basic Agglutinin in *Spodoptera frugiperda* insect cell expression system

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In this paper we report the successful expression of the winged bean basic agglutinin (WBA I) in insect cells infected with a recombinant baculovirus carrying the WBA I gene and its characterization in terms of its carbohydrate binding properties. The expressed protein appears to have a lower molecular weight than the native counterpart which is consistent with the lack of glycosylation of the former. Moreover, the expressed protein maintains its dimeric nature. Hence, a role for glycosylation in modulation of dimerization of WBA I is ruled out unlike *Erythrina corallodendron* (EcorL). Despite this the protein is active, with its sugar specificity unaltered.

KEY WORDS: Legume lectins; winged bean basic lectin; Baculovirus-insect cell expression system

INTRODUCTION

The winged bean basic lectin (WBA I), isolated from seeds of the legume *Psophocarpus tetragonolobus*, is a GalNAc specific lectin [1–3]. It is a homodimeric glycoprotein with subunit molecular weight of about 29 kDa and pI greater than 9.5. Unlike its acidic counterpart, it specifically recognizes human A and B antigen, but not the O blood group. It exhibits very distinct anomeric specificity, in that it binds the α anomer of galactose much more strongly than the β anomer [1–3]. From the crystal structure of WBA I complexed to methyl-α-galactoside, it has been proposed that the large sized D loop (one of the four that make up the legume lectin binding site) effectively precludes the binding of methyl-galactoside in its β anomeric form [4].

A general and important feature of legume lectin ligand specificity is that, of the 4 loops—A,B,C and D—that make up the binding site of these lectins, the size of the D loop appears to determine the Glc/Gal specificity of these lectins [5, 6]. In general, the Gal/GalNAc specific lectins have a longer D-loop than the Glc/Man

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ones [6]. Notably, amongst all the legume lectins known so far, WBA I has the largest D-loopl [6].

Another important feature of legume lectins is that, despite very similar tertiary structural fold, they vary widely in their modes of subunit association. For instance, the "canonical association" of concanavalin A, pea and lentil lectin [7–9], the "back-to-back" association of GSIV [10], the "open quaternary association" of peanut lectin [11] and the "hand shake" association of ECorL and WBA I [12, 4]. Small differences in amino acid composition appear to be, partly, responsible for these widely different modes of subunit association. Specifically in the case of WBA I, the amino acid sequence at the interacting surfaces, rather than glycosylation (as, perhaps, in ECorL), appear to determine its specific mode of dimer association [4, 12, 13]. Legume lectins thus not only serve as paradigms, in the study of carbohydrate recognition, but also as models for studies on the folding pathways of oligomeric proteins and the mechanisms of subunit oligomerization. Therefore, the cloning and expression of these proteins would provide the necessary tools to assess the role of specific amino acid residues in the structure of these proteins, and their mode of ligand recognition.

Earlier we had reported the cloning of WBA I by PCR amplification from genomic DNA [13]. Subsequent attempts at its over-expression in various *E. coli* expression systems have been largely unsuccessful. Though high yields and considerable ease in handling make *E. coli* the preferred system for expression of most proteins, in many instances they fail to yield active protein [14–16]. This may, either be due to specific toxicity of the expressed protein, or non-specific toxicity, where the over-utilization of the host protein synthesis apparatus may result in lowered cell viability [14–16]. Also in many instances, proteins are expressed as inclusion bodies in the cell. Though there are numerous well standardized methods for the isolation of inclusion bodies and their refolding into the native form, the final yields of active protein is often variable [17, 18]. Our attempts at expression of WBA I in *E. coli* hosts have either provided no detectable expression or low expression levels, which are present as inclusion bodies, the refolding of this has failed to yield any active protein. Hence, we decided to attempt expression in the baculovirus-insect cell system as reported here.

EXPERIMENTAL PROCEDURES

Materials

Galactose, Me-α-galactoside, Me-β-galactoside, *N*-acetylgalactosamine, glucose and mannose were procured from Sigma, St. Louis, MO. The tissue culture media, TNMFH as well as 100X antibiotic–antimycotic solution and fetal calf serum were purchased from Sigma.

Cloning of WBA I

WBA I expression in the Baculovirus system was carried out using the BAK-PAK Baculovirus expression system (Clontech Laboratories). The transfer vector

used was pBacPAK 8. The WBA I gene was PCR amplified from winged bean genomic DNA and cloned into pRSET-B [13]. Subsequently, it was subcloned in the XhoI/HindIII sites of the baculovirus transfer vector, pBacPAK 8. Henceforth the methods followed were as per the manufacturer's instructions. The recombinant baculovirus, vAcWBA I was constructed, characterized and plaque purified as described [19]. The plaques obtained after recombination were screened by dot blot, wherein after infection of the Sf9 cells with vAcWBA I for 72 hr the cells were lysed and blotted onto a H-bond paper by vacuum blotting. The blots were then washed and probed using the ^{32}PdCTP labeled WBA I fragment, excised out from the construct as template, for 3 hrs. Subsequently, they were washed, dried and exposed to a X-ray film. vAcWBA I was propagated in Sf9 cells growing in TNMFH medium supplemented with 10% fetal calf serum.

Expression of WBA I in Sf9 Cells

Cells from 35 mm culture dishes, infected with the vAcWBA I (MOI 1) were harvested 48–72 hr post infection [20]. Subsequently, the cells were harvested by centrifugation at 12,000 rpm and the pellet obtained resuspended in 150 μl PBS. The cells were lysed by forcing them through a 21-gauge syringe and the cell debris removed by centrifugation. The cell lysate was then loaded onto an amino caproyl galactosamine column and washed thoroughly with phosphate buffered saline pH 7.4 (PBS) till $A_{280\,nm} < 0.005$. Purified recombinant WBA I was eluted from the affinity matrix with 0.2 M galactose and dialysed against PBS to remove the bound sugar [21]. Expression of the protein was checked by metabolic labeling. 48 and 72 hr post infection, the Sf9 cells were incubated for one hr in methionine deficient TNMFH (Grace's insect cell culture medium supplemented with 3.33 g of yeast extract, 3.33 g of lactalbumin enzymatic hydrolysate, 0.35 g of sodium bicarbonate (all per liter) followed by a 1 hr incubation in Met$^-$ TNMFH containing 25 μCi/L–[^{35}S] methionine (1000 Ci/mol; Life Technologies) [20]. Cells were then washed in PBS and the cell pellet resuspended in sample buffer [20]. Samples were then electrophoresed on a 12% SDS PAGE, dried and subjected to direct autoradiography. Wild type *Autographa californica* nuclear polyhedrosis virus (AcNPV) infected cells were used as controls.

Hemagglutination and Hemagglutination Inhibition

The expression of WBA I was confirmed by slide agglutination of human A and B blood group [1]. Human A, B and O blood group RBCs were washed thrice with PBS and resuspended to a final 1% solution. To 20 μl serial dilution of rWBA, 20 μl of 1% RBC solution was added and agglutination checked by visual inspection under the microscope. Hemagglutination unit is defined as the reciprocal of the highest serial dilution of the protein that gives detectable agglutination. For hemagglutination inhibition, 20 μl of 2X of the above hemagglutinating unit of the protein was first preincubated with serial dilution of sugar (20 μl each) and the inhibition of agglutination checked with 20 μl RBC solutions. The sugars used for checking inhibitions were galactose, Me-α-galactoside, Me-β-galactoside, *N*-acetylgalactosamine,

glucose and mannose. Identical hemagglutination and inhibition experiments were also set up with the seed purified native lectin for comparison and quantitation.

Neutral Sugar Estimation

Total neutral sugar content was determined by the phenol sulfuric acid method using mannose as the standard [22].

RESULTS AND DISCUSSION

In the baculovirus expression virus system, the very late polyhedrin gene promoter of AcNPV is used to direct the expression of foreign genes [20, 23, 24]. Since this promoter is expressed in the late stages of the infectious viral life cycle when most of the host protein synthesis is shut down, the problems related to protein toxicity are rarely, if ever, encountered. Sf9 cells were co-transfected with the recombinant pBacPAK 8 and the specially designed BacPAK 6 viral DNA [24]. The resultant plaques were screened for recombinant virus by dot blot, using ^{32}P dCTP labeled WBA I gene as probe. Recombinant virus in the supernatant were amplified by

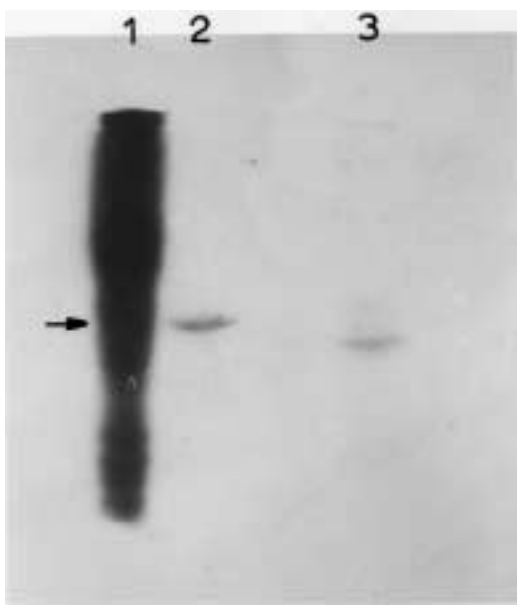

Fig. 1. Autoradiogram of the SDS PAGE of metabolically labeled cell culture lysates. Lane 1: lysate of uninfected cells; lane 2: lysate of infected cells with wild-type virus (AcNPV), the band seen is that of polyhedrin, a protein expressed in very large amount, so much so that other proteins are not seen; lane 3: lysate of cells infected with recombinant virus (vAcWBA I). Cells were harvested 72 hr post-infection. The arrow shows the expected migration of native seed WBA I (M_r 29,000).

infecting Sf9 cells at a MOI of 1.0. Expression of rWBA I was checked by metabolic labeling in 35 mm culture dishes, using [^{35}S]-methionine, 48 and 72 hr post-infection. Highest levels of expression were observed 72 hr post-infection (Fig. 1). We note that the molecular weight of the expressed protein is less than the expected 29 kDa (Fig. 2). The reason for this aberrant molecular weight is not clear, but it may be due to non-glycosylation of the expressed protein and indeed the purified rWBA I was found to be devoid of covalently linked sugars. Both the native and rWBA I eluted as dimeric species on gel filtration on a Biogel P-100 column. This indicates that the lack of glycosylation of WBA I does not compromise its dimerization ability. The purified rWBA I capable of agglutinating both, the human A and B, but not the O blood group, as observed by slide agglutination (Table 1). Moreover, agglutination was inhibited when the lectin solutions were pre-incubated with galactose, Me-α-galactoside, Me-β-galactoside and *N*-acetylgalactosamine, but not by glucose and mannose (Table 2). Both Me-α-galactoside and *N*-acetylgalactosamine were better inhibitors of agglutination than Me-β-galactoside and galactose, just as the seed protein. Notably, the values of minimum inhibitory concentrations estimated for the various sugars, were similar to that obtained for the native seed lectin (Table 2) [1].

Thus, despite its decreased molecular weight, the protein is still expressed in active form with its specificity intact. Considering the inability to obtain the protein in active form in the bacterial expression system, suggests that an appropriate

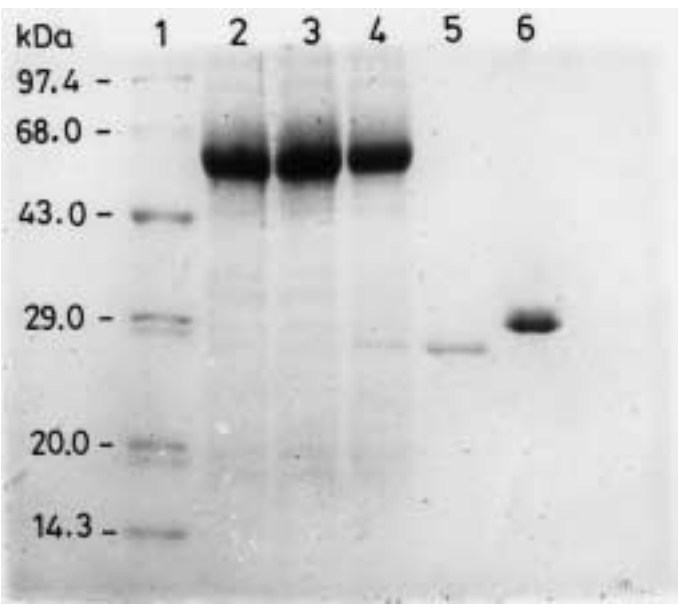

Fig. 2. 12% SDS PAGE of the infected and uninfected Sf9 cells. Lane 1 molecular weight markers; lane 2, uninfected Sf9 cells; lane 3, wild-type (AcNPV) virus infected Sf9 cells; lane 4, recombinant virus (vAcWBA I)–infected Sf9 cells, lane 5, purified recombinant WBA I and lane 6, native WBA I. The protein bands were visualized by Coomassie staining.

Table 1. Hemagglutination (by Visual Inspection under Microscope) by reWBA I.[a]

1% RBCs in PBS	rWBA I (μg/ml)	ntWBA I (μg/ml)
A-blood group	3.0	3.0
B-blood group	6.0	6.0
O-blood group	NAD	NAD

NAD, No agglutination detected even at 30 μg/ml.
[a]The native lectin, isolated from the seeds, was used as positive control. Concentration of WBA I required for observing complete agglutination are reported.

Table 2. Hemagglutination Inhibition Assay for the Minimum Concentration of Various Sugar Required to Inhibit the Agglutination of Recombinant and Native WBA I.

Sugar	rWBA I (mM)	ntWBA I (mM)
Galactose	3.2	3.2
Me-α-galactoside	0.8	0.8
Me-β-galactoside	2.7	3.0
N-acetylgalactosamine	0.75	0.7
Glucose	NI	NI
Mannose	NI	NI

NI, No inhibition observed at 200 mM of the sugars.
[a]The lectin solutions (12 μg protein) were pre-incubated with serial dilution of the sugars before addition of RBC (human A blood group). See "Experimental Procedures" for details.

"cellular" environment and/or host factors are essential for the expression of this protein in its active form. Also, this report suggests that glycosylation may not be essential for its final dimeric structure, as well as carbohydrate recognition by this protein. This is also inferred from the 2.5 Å resolution structure of WBA I, wherein the glycosylation site is seen to be far away from the inter-subunit interface [25].

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