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Trimeric glycoproteins of bean seed storage protein phaseolin were purified from baculovirus-infected insect Sf9 cells for use of structural study

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ABSTRACT

We demonstrated here insect Sf9 cells provide a useful source of a large quantity of homogeneous plant protein to be used for further structural analysis by X-ray crystallography and other biophysical probes. The seed storage protein phaseolin accumulates in common bean (*Phaseolus vulgaris* L.) as a trimeric glycoprotein in the vacuolar protein bodies of developing cotyledon. Here we characterized the post-translational modifications of phaseolin, glycosylation and trimer formation, after expression in Sf9 cells of fall armyworm (*Spodoptera frugiperda*) infected by baculovirus. When a cDNA for the mature phaseolin protein (without its own signal peptide) was placed under control of the signal peptide of viral protein GP67 in baculovirus transfer vectors pAcGP67A, phaseolin accumulated within cells at a high level (40 μg/mL). To facilitate the protein purification, six histidines were added to the carboxyl terminal of phaseolin coding sequence as a metal-chelating affinity tag. Phaseolin was extracted from Sf9 cells by 6.0 M guanidinium chloride or 4.0 M urea as a protein solubilizing agent not as a denaturant, and purified by step-wise elution from a nickel column. Phaseolin was modified by a high-mannose glycan at two potential glycosylation-sties in insect Sf9 cells as demonstrated by digestion with endoglycosidase H or peptide N-glycosidase F. Asn^{228} and Asn^{317} of two potential glycosylation-sites were converted either singly or simultaneously to Glu by site-directed mutagenesis of the cDNA. Similar amounts of wild-type and glycosylation-minus mutants were purified from Sf9 cells. Analytical equilibrium centrifugation analysis demonstrated trimer formation of both wild-type and glycosylation-minus phaseolin. The results indicate that glycosylation is not required for the protein stability or trimer formation of phaseolin.

When phaseolin was expressed under control of its own signal peptide in a second transfection vector pAcSG2, phaseolin was accumulated within cells similarly to the first constructs. However, elimination of two but not one glycosilation-sites resulted in the endoproteolytic cleavage(s) of the mature protein. Circular dichroism analysis indicated the proper secondary structure formation of phaseolin in insect Sf9 cells. Taken together, phaseolin was glycosylated, folded into the proper tertiary structure, and assembled into a trimer in insect Sf9 cells.

1. Introduction

Two major types of storage proteins in legume seeds are 7 and 11S globulins, known, respectively, as vicilins and legumins. Phaseolin is the 7S vicilin of common bean (*Phaseolus vulgaris* L.) and represents 34–50% of the total protein in mature seed. Since protein quality of phaseolin determines the economic value of bean seeds, we are interested in improving the protein quality of phaseolin using protein engineering. One of the prerequisites for this approach is a capability to purify a large quantity of homogeneous phaseolin protein and to use it for structural analysis of the engineered protein using biophysical probes.

Three-dimensional structures of phaseolin as trimeric glycoproteins were elucidated by X-ray crystallography at 3.0 and 2.3 Å resolution by Lawrence et al. [1,2]. Phaseolin polypeptide comprises two structurally similar units, each consisting of a central β-barrel ("jelly-roll" folding topology) and a peripheral α-helix-turn-helix (HTH) domain. We generated the complete tertiary structure of phaseolin based from α-carbon coordinates of

Abbreviations: CD, circular dichroism; ELISA, enzyme-linked immunosorbent assay; Endo H, endoglycosidase H; GdmCl, guanidinium chloride or guanidine HCl; HTH, helix-turn-helix; MOI, muliplicity of infection; PAGE, polyacrylamide gel electrophoresis; PB, protein bodies; PBS, phosphate buffered saline; PNGase F, peptide N-glycosidase F; and Sf9, fall armyworm (*Spodoptera frugiperda*) 9 cells.

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Lawrence et al. [1] and studied the interaction of major protein domains by molecular dynamics simulations [3,4]. We used several biophysical probes including circular dichroism (CD) to determine the structural stability of phaseolin by monitoring denaturation induced by urea, guanidinium chloride, pH changes, increasing temperature, or a combination thereof [5]. Phaseolin remained folded to a similar extent in the presence or absence of 6.0 M guanidinium chloride or 6.0 M urea at room temperature at pH 7.4. Denaturation of phaseolin occurred in 6.0 M guanidinium chloride only when the temperature was raised to 65 °C, or when pH was increased to above 8.5 or reduced to below 4.0.

We then expressed phaseolin as a part of maltose-binding fusion protein in *Escherichia coli* [6]. The non-glycosylated monomeric form of phaseolin isolated from *E. coli* denatured reversibly at 61 °C, while the glycosylated trimeric form of the protein from bean denatures irreversibly at 78 °C. It is most likely that the observed difference in protein denaturation profile is caused by post-translational modifications of phaseolin occurred within the bean plants. Thus, we are interested in expressing phaseolin in baculovirus-infected insect cells to test whether the protein is modified to the glycosylated trimer and, if so to determine how the post-translational modifications will affect the protein stability.

Phaseolin is encoded by six to eight genes per haploid bean genome and consists of nearly identical α- and β-polypeptides. The α-phaseolin (411–412 amino acids) and β-phaseolin genes (397 amino acids) share 98% identity with an exception of 18 amino acid insertions at the C-terminal end of α-phaseolin gene. Both phaseolin protein and genes have been isolated and characterized [7]. In developing bean cotyledons phaseolin is synthesized on polysome of the rough ER [8]. The nascent polypeptide enters the lumen of the ER where the signal peptide is cleaved co-translationally. The polypeptide is glycosylated by a high-mannose glycan $Glc_3Man_9GlcNac_2$, folded into the proper tertiary structure, and assembled into a trimer [8,9]. A BiP-like chaperone associates the phaseolin monomer and mediates the proper folding and trimer assembly [10–13]. The rate of trimer assembly is controled by the number of N-linked oligosaccharide chains as well as by trimming of its terminal glucose residues of $Glc_3Man_9GlcNac_2$ [14]. Phaseolin trimer is departed in small transport vesicles from the ER to the Golgi complex either by default bulk-flow or active secretion via sorting receptors [8]. The conversion of the high-mannose glycan to complex glycan was used as evidence that the protein was transported through the medial/*trans*-cisternae of the Golgi complex [13]. Glycosylated phaseolin trimer is targeted to the vacuole and packaged into the protein body where the fragmentation of phaseolin has been observed [15].

Glycosylation by a high-mannose glycan $Glc_3Man_9GlcNac_2$ might play a role in structural stability of phaseolin. Phaseolin contains two potential glycosylation-sites (Asn-X-Thr) at position Asn^{228} and Asn^{317} located at the surface-exposed region of the carboxyl β-barrel [1,2]. However, phaseolin is not uniformly glycosylated producing at least two glycoforms. One glycoform contains high-mannose glycans at both positions, $Man_7(GlcNAc)_2$ at Asn^{228} and $Man_9(GlcNAc)_2$ at Asn^{317}. A second glycoform contains a complex glycan Xyl-$Man_3(GlcNAc)_2$ at Asn^{228} and no glycosilation at Asn^{317} [9]. The extent of glycosylation varies also among bean cultivars. The glycosylation-site knock-out experiment in transgenic tobacco has demonstrated that glycans are not required for proper transport to protein bodies [16]. However, it was difficult to determine using transgenic tobacco whether the non-glycosylated proteins are less stable than glycosylated wild-type protein.

Phaseolin has been expressed in a number of heterologous plants, including sunflower [17], tobacco [18–20], and rice [21]. However, the production of transgenic plant is time-consuming, and it has been difficult to purify from mature seeds a large quantity of the homogeneous protein products for structural analysis. Thus, we chose heterologous protein expression systems of bacteria *E. coli* and baculovirus-infected insect Sf9 cells [22]. When the amino acid substitution mutations were introduced to enhance the methionine content of phaseolin, the mutations did not alter the structural stability of the protein after expression in *E. coli* as a non-glycosylated monomer. Here we report use of a baculovirus-transfected insect Sf9 cells to characterize post-translational modifications of phaseolin, i.e. glycosylation and trimer formation, and further to determine the effect of glycosylation on the structural stability and trimer formation of phaseolin.

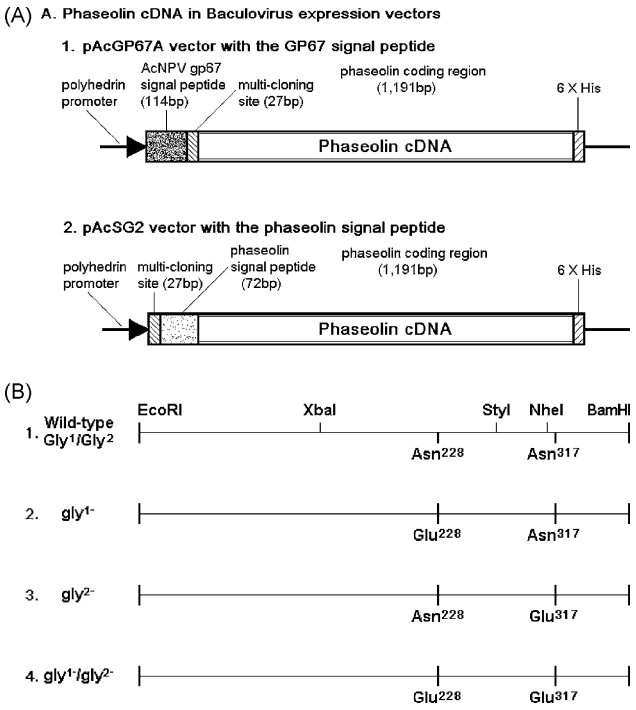

Fig. 1. Schematic diagram of phaseolin cDNA in two baculovirus transfer vectors pAcGP67A and pAcSG2 (A), and two potential N-glycosylation-sites in the wild-type phaseolin and a single or double mutations to remove the glycosylation-sites (B). A1, a phaseolin cDNA encoding the mature protein (without its own signal peptide) was inserted to EcoRI/BglII sites of a transfer vector pAcGP67A and the protein was expressed under control of the polyhedrin promoter and the gp67 signal peptide. As a result of the constructions, nine amino acid residues were added to the amino terminal of the phaseolin mature protein. Six histidine residues were introduced to the carboxyl terminal and used as an affinity tag to facilitate protein purification using a nickel column. A2, a phaseolin cDNA for the signal peptide and mature protein was introduced to the XhoI/BglII sites of a second transfer vector pAcSG2. Six histidine residues were also added to the carboxyl terminal as an affinity tag for protein purification. (B) Two potential N-glycosylation-sites at the residues Asn^{228} and Asn^{317} in the wild-type phaseolin, and a single or double mutations of the glycosylation-sites by site-directed mutagenesis of cDNA to convert the asparagine to glutamate codon. B1, wild-type cDNA Gly^1/Gly^2; B2, gly^{1-} mutation; B3, gly^{2-} mutation; B4, gly^{1-}/gly^{2-} double mutations.

2. Materials and methods

2.1. Plasmid DNAs and phaseolin cDNA modifications

Baculovirus transfer vectors pAcGP67A and pAcSG2 (Fig. 1A), and BaculoGold™DNA were purchased from BD Bioscience-PharMingen (San Diego, CA). A full-length cDNA for phaseolin in pPhcDNA 31 was kindly provided by J.L. Slightom, University of Wisconsin-Madison.

Previously, we introduced a BglII site by site-directed mutagenesis [23] in the 5′-non-coding region of cDNA 15 bases upstream of the initiation codon ATG [24]. Four unique restriction sites had been also introduced to facilitate the construction for methion-