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High-level expression and purification of recombinant wheat germ agglutinin in *Rachiplusia nu* larvae

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ABSTRACT

Wheat germ agglutinin (WGA) is a homodimeric lectin stabilized by non-covalent interactions. Each monomer of 171 residues, which has a complex structure with 16 disulfide bridges, determines two sites for the specific binding of *N*-acetyl-D-glucosamine and one for the specific binding of *N*-acetylneuraminic acid. Because of these folding requirements, the production of high yields of recombinant WGA is still not possible and its extraction from wheat germ is the only source for commercial purposes. This work reports for the first time the expression of WGA isolectin A (WGA A), using a baculovirus expression system in Sf9 cells and *Rachiplusia nu* larvae. High levels of recombinant WGA A were obtained in both cases, especially in *R. nu*, where yields reached 346.6 ± 88.5 μg/g of larvae. Also, an integrated purification method was developed based on aqueous two-phase separation coupled to affinity chromatography using chitosan mini-spheres. The recombinant WGA A was able to recognize ovoalbumin sugar moieties, cross-react with anti-WGA serum and agglutinate human red blood cells, and showed the same behavior as that of commercial WGA in SDS-PAGE and RP-HPLC analyses.

1. Introduction

Lectins are ubiquitous proteins that reversibly interact with specific carbohydrate moieties. They have been isolated and characterized from different sources such as bacteria, viruses, plants, vertebrates and invertebrates [1–3]. Lectins are useful tools in different biotechnological applications due to their specific carbohydrate-binding capacity. Many lectins have antiviral, antifungal, antibacterial and antinematode activity [1,3–6]. Several lectins are proposed as potential tools in cancer diagnosis, since various tumor cells have an aberrant glycosylation pattern in their membrane [7]. In addition, lectins are used as affinity ligands immobilized on chromatographic supports for the purification of specific glycoproteins [8–10].

Wheat germ agglutinin (WGA) is a lectin whose isolation yields a mixture of three isolectins, WGA A, WGA B and WGA D. Each isolectin consists of two identical subunits of 171 residues forming an active dimer of 35 kDa which is stabilized by non-covalent interactions [11,12]. Each monomer has a complex structure with 16 disulfide bridges that determines two sites for the specific binding of *N*-acetyl-D-glucosamine (GlcNAc) and one for the specific binding of *N*-acetylneuraminic acid (Neu5Ac) [11,13]. WGA is slightly soluble in water or neutral buffers and soluble in acid buffers without any loss of activity [10,12–14]. In the last years, WGA has been used as a "recognition" moiety in glycan-targeted drug delivery applications, since WGA mediates binding and enhances the drug uptake into diseased cells [15–17]. Also, WGA has successfully been tested as a biomolecular "coating" material of scaffolds in tissue engineering, with the aim of improving cell adhesion and promoting tissue ingrowths [18].

Due to the structural complexity of WGA, its production in high yield as a recombinant protein for commercial purposes is still not possible. Its overexpression in prokaryotes might lead to inclusion bodies, thus requiring a further renaturalization step, which could be inefficient for the correct establishment of disulfide bridges [2]. On the other hand, WGA B has been recombinantly expressed and purified from *Saccharomyces cerevisiae*, with low yields [19]. Therefore, the main source of WGA is still wheat germ. Its extraction involves the use of approximately 6 L of hexane and 10 L of diluted hydrochloric acid per kilogram of wheat germ [10,13]. Then, several purification steps are needed to accomplish the final adsorption step of WGA to a chromatographic support made of chitin or chitosan [13,14]. The yield of this process is approximately 200–400 mg of WGA from 1 kg of wheat germ.

The downstream processing of WGA not only is a high-cost and time-consuming process, but also implies the use of an edible source as a starting material and large volumes of organic solvents. Therefore, in order to minimize the negative environmental impact,

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it is interesting to explore new strategies for the production of WGA coupled to low-cost purification methods.

The baculovirus expression vector system (BEVS) is a versatile tool for the production of recombinant proteins using insect cells or insect larvae. Usually, BEVS is used when the expression of a protein has failed using bacteria or yeast. BEVS allows achieving correct protein folding, disulfide bond formation and post-translational modifications. This system uses a recombinant *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) and insect cell lines, such as those from *Spodoptera frugiperda* (Sf21, Sf9) and *Trichoplusia ni* (Tn-5) [20]. High levels of heterologous proteins are obtained when using insect cell lines. However, the scaling-up of recombinant protein production in insect cell culture is expensive. The costs of commercial cell-culture medium have become too high for very large volumes and a considerable investment in highly technical equipment (e.g. bioreactors) is required. Despite these facts, BEVS versatility allows using insect larvae (Lepidoptera, Noctuidae family) for fast and low-cost production of a recombinant protein. The use of larvae as "biofactories" could reduce the manufacturing costs up to four hundred times in comparison with insect cell cultures [21–24]. On the other hand, the cost of the downstream processing step could increase significantly when using insect larvae as expression hosts, especially when several purification steps are required [25–27]. Therefore, there is a growing interest in developing new low-cost integrated purification methods coupled to the large-scale production of recombinant proteins by using insect larvae. This approach using lepidopteran larvae may decrease the overall costs of protein production and would allow BEVS to become a cost-effective and competitive scaling-up platform for recombinant protein production.

Some plant lectins have been successfully expressed using BEVS and insect cells with yields of around 1–10 mg/L [28–31]. These complex proteins have only been expressed using insect cells but not using insect larvae. Here we report for the first time the recombinant expression of WGA A in Sf9 cells and *Rachiplusia nu* larvae, a destructive plague that affects economically important crops. Also, an integrated purification method was developed based on aqueous two-phase separation coupled to affinity chromatography using mini-spheres of chitosan, a linear copolymer of randomly distributed D-glucosamine (GlcN) and GlcNAc.

2. Materials and methods

2.1. Analytical reagents

The molecular weight standards PageRuler™ Prestained (Thermo Scientific, Rockford, IL, USA) and BenchMark™ Protein Ladder (Invitrogen, Gaithersburg, MD, USA) were used for SDS-PAGE. The nitrocellulose membrane and reagents for chemiluminescence detection were from GE Healthcare (Piscataway, NJ, USA). The serum anti-WGA from mouse was obtained from the Cátedra de Inmunología- IDEHU, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires (Buenos Aires, Argentina) as a technical service. Commercial WGA was used as the immunogen. The rabbit anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody was obtained from DAKO (Glostrup, Denmark). The Quick start™ Bradford reagent for total protein determination was obtained from BioRad (Hercules, CA, USA). Cellfectin® and 3,3′,5,5′-tetramethylbenzidine (TMB) reagents were from Invitrogen™. Chitosan (medium molecular weight), Triton X-114 and GlcNAc were from Sigma–Aldrich (St. Louis, MO, USA) and epichlorohydrin was from Fluka Analytical (Buchs SG, Switzerland). Commercial WGA from *Triticum vulgaris* was from Megazyme (Bray Wicklow, Ireland). Ovoalbumin was from Ovaport International S.A. (Buenos Aires, Argentina). All other reagents were of analytical grade.

2.2. Molecular biology

The DNA sequence of WGA A (GenBank accession no. P10968) was codon-optimized for baculovirus expression, synthesized and cloned in the pUC57 vector by GenScript (Piscataway, NJ, USA). The transfer vector pAcGP67B was from BD Bioscience and the baculovirus bAcGOZA [32] was gently donated by Dr. Oscar Taboga (INTA Castelar, Buenos Aires, Argentina). The insect cell line IPBL-Sf9 from *S. frugiperda* (Sf9) was purchased from Asociación Banco Argentino de Células (Pergamino, Buenos Aires, Argentina). The Sf900 II insect cell culture medium and the antibiotic and antimycotic solutions were from Invitrogen™. The fetal bovine serum (FBS) was from Nutrientes Naturales S.A. (Buenos Aires, Argentina).

2.3. Recombinant baculovirus construction

The DNA sequence of WGA A (636 nucleotides) cloned in the pUC57 vector was subcloned in the pAcGP67B transfer vector using the primers 5′CAAGGATCCCAAAGATGTGGCGAACAAG 3′ and 5′GCCGAATTCTTAGCCGTCACAGCCGCC3′ and the BamHI and EcoRI cloning sites, which rendered the construction pAcGP67B-WGA. This construction was cotransfected with bAcGOZA using Cellfectin® (cationic liposome method) in Sf9 cells [33] to obtain the baculovirus named bAcGOZA-WGA. This baculovirus presents the WGA A gene under the control of the polyhedrin viral promoter and keeps the polyhedrin gene under the p10 viral promoter.

2.4. Expression of WGA A in insect cell cultures

Sf9 suspension cultures (2×10^6 cells/mL) grown in Sf900 II medium supplemented with 1% of FBS were infected with bAcGOZA-WGA at a multiplicity of infection (MOI) of 0.5, 2 and 10. Samples of 1 mL were collected each day post-infection (dpi) to quantify the recombinant production of WGA by ELISA and for protein analysis by Western blot. Briefly, the culture medium was separated from the cells by centrifugation at $10{,}000 \times g$ for 10 min. The pellet and the supernatant were stored at −20 °C until further experiments. For control purposes, Sf9 suspension cultures infected with a *wild type* baculovirus was used.

2.5. WGA A extraction from infected cells

The cell pellet was resuspended in lysis buffer containing 50 mM HCl, 150 mM NaCl and 1 mM of phenylmethylsulfonyl fluoride (PMSF), pH 1.3 (1 mL of lysis buffer was used per 2×10^7 cells). After incubation at 4 °C for 30 min, the cellular debris was separated by centrifugation at $12{,}000 \times g$ for 30 min at 4 °C. The pellet was discarded and the supernatant was stored at −20 °C until further experiments.

2.6. Detection of recombinant WGA A by ELISA

WGA A was quantified according to Vincenzi et al. [34] with minor modifications. Flat-bottomed 96-well microtiter plates (*Nunc Maxisorp®*, *Thermo Fischer Scientific*, Waltham, MA, USA) were coated with 50 μL of a 200 μg/mL ovoalbumin solution in 50 mM carbonate buffer (pH 9.6). For WGA A detection, 50 μL of the anti-WGA serum (diluted 1:1000 in phosphate buffer solution (PBS) with 1% of bovine serum albumin (BSA)) and 50 μL of secondary antibody (diluted 1:5000 in PBS-BSA) were used. For peroxidase detection, 75 μL of TMB was added to each well and after 15 min at room temperature the reaction was stopped with 75 μL of H_2SO_4 0.18 M. Plates were read using an ELISA EZ Read 400 Microplate

Reader from Biochrom (Milton Road, Cambridge, UK) at a wavelength of 450 nm. Samples were diluted using PBS–BSA. The amount of recombinant WGA A was compared with a standard curve of commercial WGA, ranging from 7.8 to 1000 ng/mL. All determinations were performed in triplicate and results are expressed as the average ± standard deviation.

2.7. Western blot analysis

Samples from the supernatants and pellet were quantified by the Bradford assay [35] using the Quick start™ Bradford reagent, and 10 μg of total protein from each dpi was loaded and separated in a 15% SDS-PAGE. Proteins were blotted onto a nitro-cellulose membrane. The membrane was blocked with 3% skim milk in PBS, pH 7.0, for 1 h at room temperature, and then incubated with anti-WGA serum as primary antibody for 1 h at room temperature (1:1000 diluted in PBS with 0.3% skim milk). After three washes of 15 min each with PBS-0.05% Tween 20, the membrane was incubated with an HRP-conjugated secondary antibody (1:1500) for 1 h at room temperature, washed again and revealed by chemiluminescence.

2.8. Affinity-matrix preparation

Chitosan mini-spheres (1–2 mm of diameter) were prepared according to Baieli et al. [10]. For the cross-linking reaction, a 250 mM epichlorohydrin solution was prepared in distilled water and the pH was adjusted to 10.0 with 1 M NaOH. Cross-linking was performed at a ratio of 25 mL epichlorohydrin solution per 15 g matrix for 4 h at 60 °C. Finally, the mini-spheres were washed with distilled water to remove residual epichlorohydrin. Hereafter, the chitosan matrix cross-linked with epichlorohydrin 250 mM is named CH250.

2.9. RP-HPLC

RP-HPLC was performed on a Shimadzu LC-20AT system. A 218TP54 Vydac column (4.6 mm × 25 cm, Separations Group, Hesperia, CA, USA) was used to analyze all the samples from the purification process. The RP-HPLC running conditions were according to Baieli et al. [10]. The injection volume was 50 μL and the concentration of WGA in the samples (upper aqueous phase, eluted WGA A and standard WGA) were around 100–125 μg/ml.

2.10. WGA A agglutinating activity

WGA agglutinating activity was estimated on A^+ human red blood cells. The activity of WGA was determined according to a modification of the procedure described by Zeng and Ruckenstein [13]. The pure WGA solution and samples were serially diluted (1:2) with PBS. The agglutination assay was made by placing 100 μL of pure WGA or sample in a microplate (96 wells with U-shape) with 50 μL of 2% suspension of red cells in PBS. Agglutination was determined after 1 h at room temperature.

2.11. Larvae infection

Larvae of *R. nu* were obtained from AgIdea S.A (Pergamino, Buenos Aires, Argentina) and reared individually in standard six-well plates on an artificial diet. Second-instar larvae were maintained at 27 °C in a 70% humidified chamber, with a 16:8 photoperiod (light:dark). A phenol-free diet was used in all oral infection experiments [36]. bAcGOZA-WGA polyhedra were collected and counted at 5 dpi from a previously infected cell culture, as described by O'Reilly et al. [33]. For oral infection, larvae of approximately 50 mg were starved for 24 h and then fed with a diet containing 1×10^6 polyhedra of bAcGOZA-WGA. After 24 h, larvae were fed normally with a diet without polyhedra. Three days post-oral infection, larvae were harvested and stored at −80 °C until further experiments. Larvae were then homogenized using 2.5 mL of larva lysis buffer (50 mM HCl, 150 mM NaCl, 4% Triton X-114 and 1 mM PMSF, pH 1.3) per gram of larvae (~6 larvae). For larvae disruption, a mortar was used, and in the case of large volumes (>10 mL) an Omni mixer homogenizer (Omni Macro International, Kannesaw, GA, USA) was used. For control purposes, a homogenate of larvae infected with *wild type* baculovirus was used. All determinations were performed in triplicate and results are expressed as the average ± standard deviation.

2.12. Aqueous two-phase separation induced by temperature

To induce the two-phase separation, larvae homogenates with 4% of Triton X-114 were incubated at 30 °C for 5 min. Then, samples were centrifuged at 10,000 × *g* for 10 min at 24 °C. The final volume ratio after separation induced by temperature was 12:1 (upper phrase: lower phrase). The upper aqueous phase was used for purification of recombinant WGA A.

2.13. Affinity-batch purification

For purification of WGA A, samples were adjusted to pH 5.0 for adsorption to the CH250 matrix using the batch mode. The CH250 matrix was equilibrated using 50 mM acetic acid buffer with 150 mM NaCl (pH 5.0). Adsorption of recombinant WGA A was performed using 25 mg of chitosan mini-spheres (CH250) per 1 mL of sample (cells or larvae extract). The suspension was mechanically stirred for 16 h at 24 °C. Then, after discarding the supernatant, the matrix was washed four times using 50 mM acetic acid buffer with 150 mM NaCl (pH 5.0) for 1 h each. Different eluents (1 M acetic acid, pH 2.5; 1 M GlcNAc dissolved in 50 mM acetic acid buffer, pH 3.0; 1 M GlcNAc dissolved in 50 mM acetic acid buffer, pH 5.0; and 1 M GlcNAc dissolved in 50 mM Tris–HCl buffer, pH 8.5) were tested overnight at 24 °C for desorption of the recombinant WGA A. WGA A was quantified by ELISA and RP-HPLC. Centrifugal filters (Millipore, MA, USA) or PD-10 desalting columns (GE Healthcare, NJ, USA) were used to condition the elution samples. Total protein was quantified by the Bradford assay and 15 μL from each purification step was loaded and separated in a 15% SDS-PAGE for Coomassie Blue or silver staining [37] and Western blot. The purification factor was calculated as the ratio between the amount of WGA A relative to the total protein content (μg WGA A/mg protein) in the eluate after the purification step, and that of the initial extract.

3. Results and discussion

3.1. Expression of recombinant WGA A using Sf9 cells

To determine the expression and localization of recombinant WGA A, Sf9 cells were infected with bAcGOZA-WGA using a MOI of 0.5. Then, supernatants and cell pellets were analyzed at different dpi by Western blot using anti-WGA serum. The WGA A gene was present in the bAcGOZA-WGA genome under the control of the polyhedrin viral promoter (Fig. 1B). The endogenous WGA A signal for secretion in wheat germ was replaced by the GP67 viral signal sequence. Also, the endogenous signal for post-translational vacuole localization in wheat germ was removed (Fig. 1A). The GP67 signal is commonly used to secrete and localize the recombinant protein in the culture medium using BEVS [33].

Fig. 2 shows the analysis of WGA A localization in Sf9 cell culture infected with bAcGOZA-WGA using Western blot. Although the WGA A gene had the GP67 viral signal sequence for protein

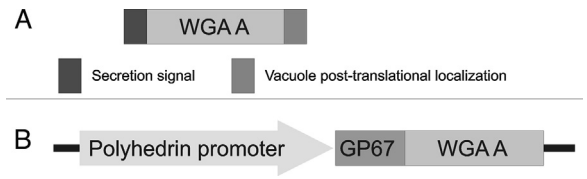

Fig. 1. The original WGA A gene sequence in the pUC57 vector (A) and the final construction in the bAcGOZA-WGA baculovirus genome (B). The endogenous WGA A signal for secretion in wheat germ was replaced by the GP67 viral signal sequence. The endogenous WGA A signal for post-translational vacuole localization in wheat germ was removed. The WGA A gene was present in the bAcGOZA-WGA genome under the control of the polyhedrin viral promoter.

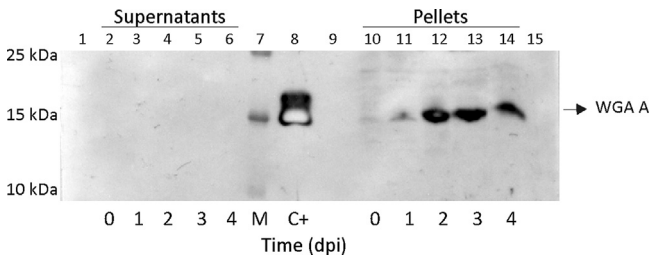

Fig. 2. Analysis of recombinant wheat germ agglutinin (WGA A) localization in supernatants and cell pellets by Western blot. Lanes 2–6 and lanes 10–14, expression of WGA A in supernatants and cell pellets, respectively, at 0, 1, 2, 3 and 4 days post-infection (dpi) with the baculovirus bAcGOZA-WGA using a multiplicity of infection (MOI) of 0.5. Lane 7, protein ladder (M). Lane 8, commercial WGA purified from wheat germ (C+). Lanes 1, 9 and 15 – empty.

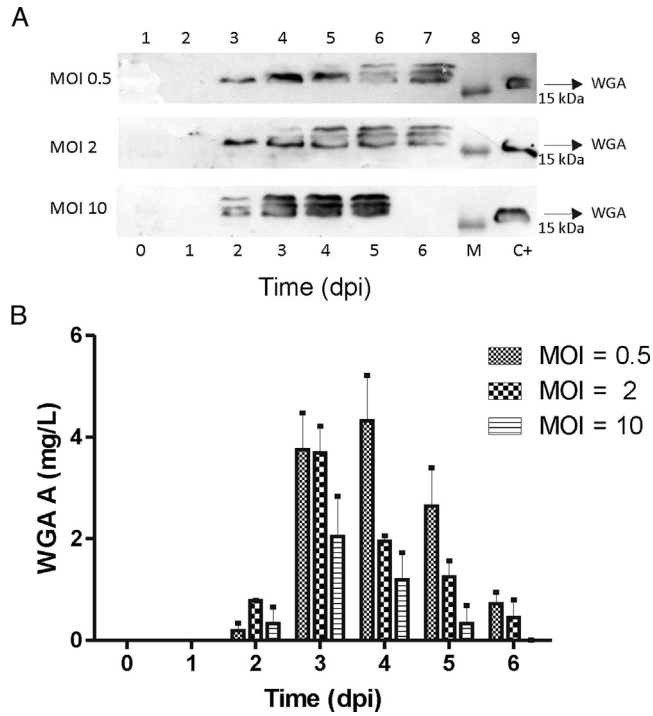

Fig. 3. Expression kinetics of recombinant wheat germ agglutinin (WGA A) in Sf9 cell pellets determined by Western blot (A) and ELISA (B). (A) Lanes 1–7, expression of WGA A in cell pellets at 0, 1, 2, 3, 4, 5 and 6 days post-infection (dpi) with the baculovirus bAcGOZA-WGA using multiplicity of infections (MOIs) of 0.5, 2 and 10. Lane 8, protein ladder. Lane 9, commercial WGA purified from wheat germ. (B) WGA A quantification at 0, 1, 2, 3, 4, 5 and 6 dpi using MOIs of 0.5, 2 and 10, with the baculovirus bAcGOZA-WGA. The amount of recombinant WGA A was determined by ELISA.

secretion, the recombinant protein was found in cell pellets and had the same electrophoretic mobility in SDS-PAGE as commercial WGA. During baculovirus infection, several viral proteins are glycosylated with GlcNAc residues and are localized in the membrane of the infected cell [38]. Since WGA has affinity for GlcNAc and Neu5Ac residues, the recombinant WGA A possibly interacted with these glycosylated viral proteins and kept bound to the cell membrane. To maximize WGA A extraction from cell pellets, the cell lysis buffer used was 50 mM HCl, 150 mM NaCl and 1 mM PMSF, pH 1.3. When the pH was below 4.0, WGA was mainly in its monomeric form and did not interact with GlcNAc or Neu5Ac residues. Interestingly, when the pH was adjusted above 4.0, WGA was mainly in its dimeric form and was able to interact again with GlcNAc and Neu5Ac residues [10,13].

Fig. 3 shows the expression kinetics of WGA A in Sf9 cell pellets determined by Western blot (Fig. 3A) and quantified by ELISA (Fig. 3B) using different MOIs. At 4 dpi and a MOI of 0.5, the yield was 4.3 ± 0.9 mg/L of WGA A. Other plant lectins have been previously expressed using BEVS and insect cell lines, with similar yields (around 1–10 mg/L) [28–31]. WGA B, which has 95% of homology with WGA A, has been previously expressed using *S. cerevisiae* as host, but low-yields (150–200 μg/L) were obtained [19]. The ELISA used was based on the affinity of WGA to the sugar moieties of ovoalbumin, which allowed us to obtain indirect information about the ability of recombinant WGA A to recognize GlcNAc and Neu5Ac residues [34]. The analysis of WGA A by Western blot (Fig. 3A) showed the presence of a heterogeneous recombinant product (three bands are observed) at 5 and 6 dpi using a MOI of 0.5, at 4, 5 and 6 dpi using a MOI of 2 and at any time post-infection using a MOI of 10. In all these cases, low quantities of WGA A able to interact with the sugar residues of ovoalbumin were detected by ELISA (Fig. 3B). The production of high levels of the recombinant protein with the strong baculovirus polyhedrin promoter and the shutdown of host protein synthesis during baculovirus infection in insect cells lead to a limitation in the supply of secretory assistance proteins [33,39,40]. For example, in late stages of the baculovirus infection, the signal peptide might remain attached to the polypeptide indicating improper processing in the secretory pathway [39]. Also, the activity of the protein disulfide isomerase (an endoplasmic reticulum enzyme that catalyzes the oxidation, reduction and isomerization of disulfide bonds) might be not enough to correctly fold the large quantity of WGA A produced (each monomer of WGA A has 16 disulfide bridges) [41,42]. These might be possible explanations for the heterogeneous WGA A product observed by Western blot (Fig. 3A) and its lower interaction with the sugar residues of ovoalbumin detected by ELISA (Fig. 3B). At earlier post-infection times using MOI 0.5, this heterogeneity was not observed. Therefore, for further purification experiments, the cell pellet of WGA A expression at 4 dpi using a MOI of 0.5 was collected.

3.2. Purification of WGA A

The cell pellet lysate was adjusted to pH 5.0 and used directly for WGA A adsorption to the CH250 matrix in the batch mode. Table 1 shows the purification process of WGA A using the CH250 matrix. The concentration of WGA A in the cell pellet lysate was ~4 mg/L and its adsorption to the CH250 matrix was almost complete (>93%). Different eluents were selected according to Baieli et al. [10] and tested to evaluate the desorption of WGA A from the CH250 matrix (Table 1). In the presence of 1 M GlcNAc, the elution of WGA A increased as the pH decreased. The same effect has been previously reported by Zeng and Ruckenstein [13] and Baieli et al. [10]. Using 1 M acetic acid and 1 M GlcNAc pH 3.0 as eluents, 62.0 ± 4.0% and 59.4 ± 4.1% of WGA A were respectively eluted from CH250 (Table 1).

Fig. 4 shows the SDS-PAGE and Western blot analysis of the purification process using 1 M acetic acid as the eluent. Around 60% of WGA A was recovered directly from the cell pellet lysate,

Table 1
Purification process of recombinant wheat germ agglutinin (WGA A) from Sf9 cells using the chitosan matrix cross-linked with epichlorohydrin 250 mM (CH250).

Matrix	Adsorbed WGA A (%)	Eluent	Eluted WGA A (%)	Purification factor
CH250	93.3 ± 0.4	1 M acetic acid, pH 2.5	62.0 ± 4.0	45
	94.7 ± 0.2	1 M GlcNAc, pH 3.0	59.4 ± 4.1	43
	94.1 ± 0.8	1 M GlcNAc, pH 5.0	22.4 ± 3.8	20
	94.6 ± 0.2	1 M GlcNAc, pH 8.5	7.4 ± 3.0	2

WGA A concentration is ~4 mg/L.

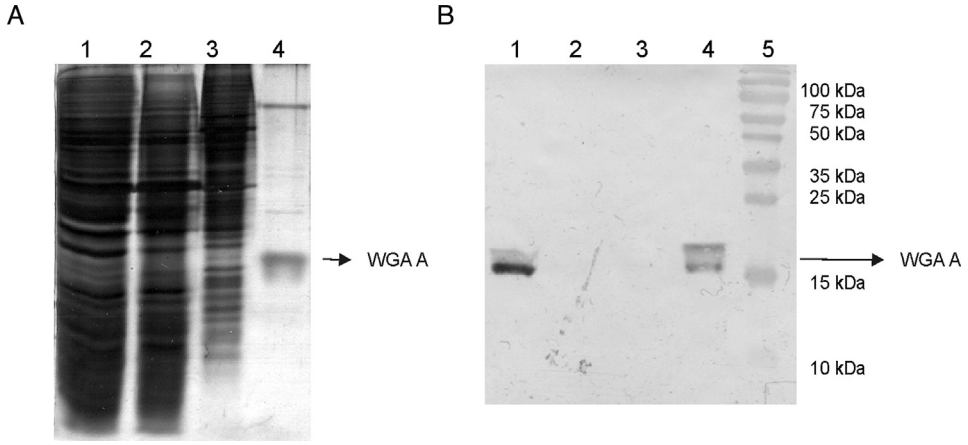

Fig. 4. SDS-PAGE (A) and Western blot (B) analysis of the recombinant wheat germ agglutinin (WGA A) purification process using the chitosan matrix cross-linked with epichlorohydrin 250 mM (CH250). Lane 1, cell pellet lysate. Lane 2, cell pellet lysate after WGA A adsorption to the CH250 matrix. Lane 3, first washing step after WGA A adsorption. Lane 4, WGA A eluted from the CH250 matrix using 1 M acetic acid. Lane 5, protein ladder. SDS-PAGE was silver-stained.

only with a step of pH adjustment, using the CH250 matrix. In one purification step, 75% of purity according to gel densitometry analysis was obtained (Fig. 4A, lane 4). The identity of the purified WGA A was also confirmed by Western blot (Fig. 4B, lane 4) and RP-HPLC (data not shown).

3.3. WGA A production in R. nu larvae

According to the expression kinetics of recombinant WGA A using *R. nu* larvae (data not shown), the larval homogenate was processed at 3 dpi and then treated using two-phase aqueous separation induced by temperature. Fig. 5A shows WGA A yield in *R. nu* larvae and the partition of WGA A in the aqueous two-phase system after induction by temperature, quantified by ELISA. The recombinant WGA A produced in *R. nu* larvae was able to recognize sugar residues from ovoalbumin in the ELISA assay. It was possible to extract 346.6 ± 88.6 μg of WGA A per gram of larvae (5–6 larvae) infected with bAcGOZA-WGA. Approximately 5–6 infected larvae produced the same amount of WGA A as that of 80 mL of Sf9 cell culture (1.6×10^8 cells).

To clarify and partially purify the homogenate for further purification purposes, an aqueous two-phase system was used. The lower phase was enriched in the detergent Triton X-114 and WGA A partitioned mostly in the upper phase. This allowed obtaining 222.9 ± 67.4 μg per gram of larvae (Fig. 5A). Probably a minor fraction of WGA A was lost associated with the larvae debris after centrifugation. The analysis of WGA A by Western blot showed the presence of a homogeneous recombinant product with an electrophoretic mobility in SDS-PAGE similar to that of commercial WGA (Fig. 5B). The upper aqueous phase was adjusted to pH 5.0 and used directly for purification of WGA A using the CH250 matrix.

Table 2 shows the WGA A purification process using the CH250 matrix determined by ELISA. The concentration of WGA A in the upper aqueous phase was ~100 mg/L and its adsorption to the CH250 matrix was almost complete (>95%). According to the results

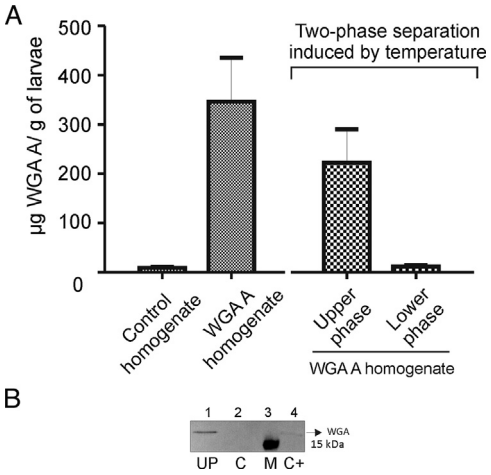

Fig. 5. Recombinant wheat germ agglutinin (WGA A) yield using *Rachiplusia nu* larvae and aqueous two-phase separation induced by temperature, determined by ELISA (A) and Western blot (B). A) ELISA quantification of WGA A expressed in *Rachiplusia nu* infected with bAcGOZA-WGA (WGA A homogenate). For control purposes, a homogenate of larvae infected with *wild type* baculovirus was used. B) Analysis of aqueous two-phase separation by Western blot. Lane 1, upper phase (UP). Lane 2, upper phase using *wild type* baculovirus as control (C). Lane 3, protein ladder (M). Lane 4, commercial WGA purified from wheat germ (C+).

shown in Table 1, 1 M acetic acid was selected as the eluent for the purification of WGA A using *R. nu* larvae. A global yield of 80% was obtained with a purification factor of 74 (Table 2).

Fig. 6 shows the SDS-PAGE and Western blot analysis of the purification process using larvae. In one purification step, 75% of purity according to gel densitometry analysis was obtained (Fig. 6A, lane 4). The purification method developed based on aqueous two-phase separation coupled to affinity chromatography using CH250 allowed purifying around 180 μg of WGA A per gram of infected larvae (5–6 larvae). The use of chitosan mini-spheres (1–2 mm

Table 2
Purification process of recombinant wheat germ agglutinin (WGA A) from *R. nu* larvae using the chitosan matrix cross-linked with epichlorohydrin 250 mM (CH250).

Matrix	Adsorbed WGA A (%)	Eluted WGA A (%)	Yield (%)	Purification factor
CH250	96.7 ± 3.4	83.2 ± 7.1	80	74

WGA A concentration is ~100 mg/L.

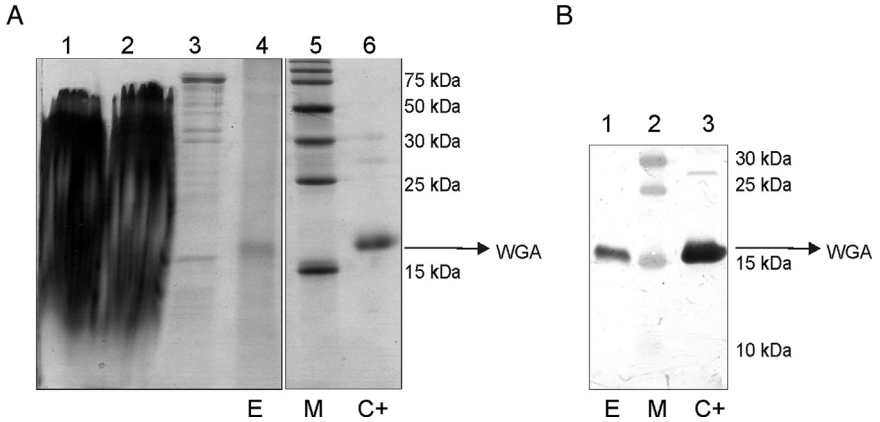

Fig. 6. SDS-PAGE (A) and Western blot (B) analysis of the recombinant wheat germ agglutinin (WGA A) purification process using the chitosan matrix cross-linked with epichlorohydrin 250 mM (CH250). (A) SDS-PAGE stained with Coomassie Blue. Lane 1, upper phase from the two-phase aqueous separation system using homogenate of *Rachiplusia nu* expressing WGA A. Lane 2, upper aqueous phase after WGA A adsorption to the CH250 matrix. Lane 3, first washing step after WGA A adsorption. Lane 4, WGA A eluted from the CH250 matrix using 1 M acetic acid (E). Lane 5, protein ladder (M). Lane 6, commercial WGA (0.5 mg/mL) purified from wheat germ (C+). All lanes belong to the same gel. (B) Western blot using anti-WGA serum. Lane 1, WGA A eluted from the CH250 matrix using 1 M acetic acid (E). Lane 2, protein ladder (M). Lane 3, commercial WGA purified from wheat germ (C+).

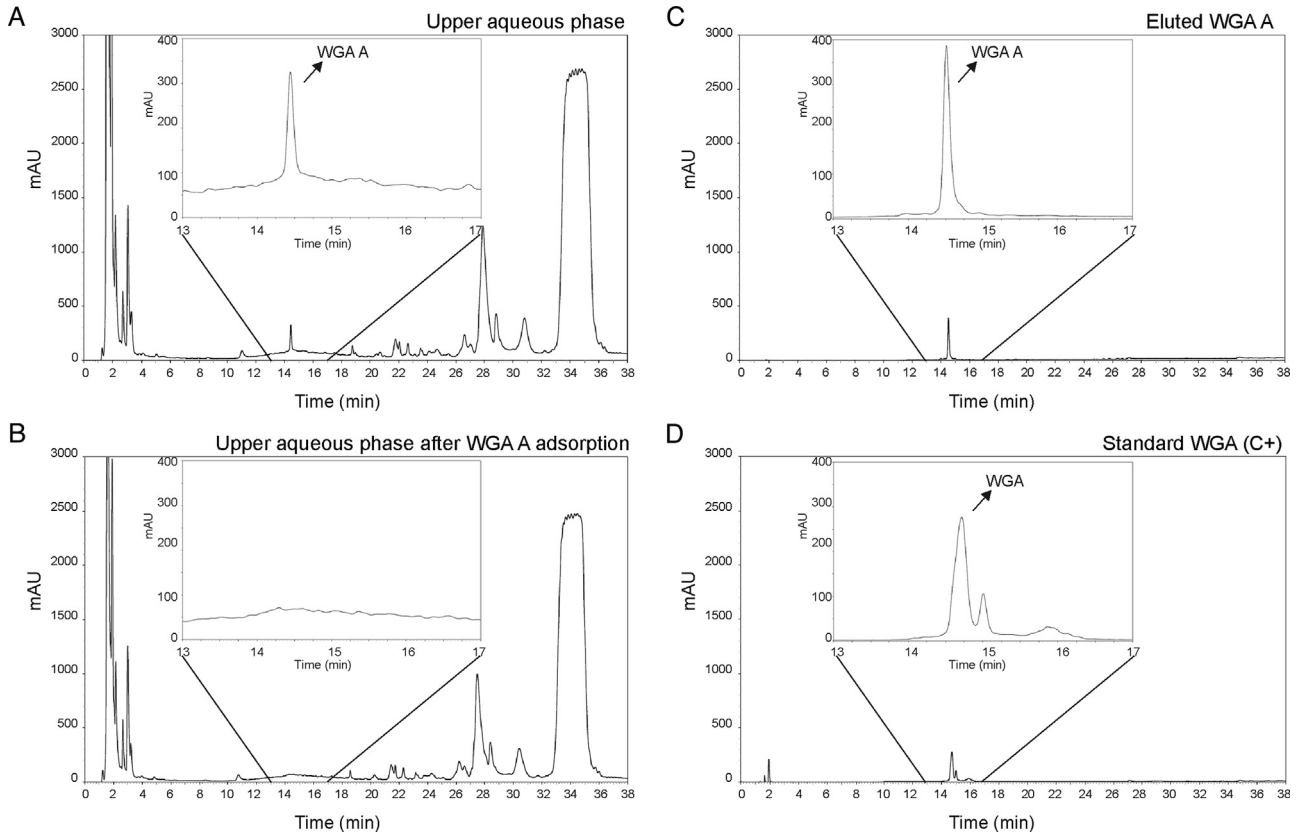

Fig. 7. RP-HPLC patterns of samples from the recombinant wheat germ agglutinin (WGA A) purification process. (A) Upper phase from the aqueous two-phase separation system using *Rachiplusia nu* homogenate expressing WGA A. (B) Upper aqueous phase after WGA A adsorption to the chitosan matrix cross-linked with epichlorohydrin 250 mM (CH250). (C) WGA A eluted from the CH250 matrix using 1 M acetic acid. (D) Commercial WGA purified from wheat germ (C+).

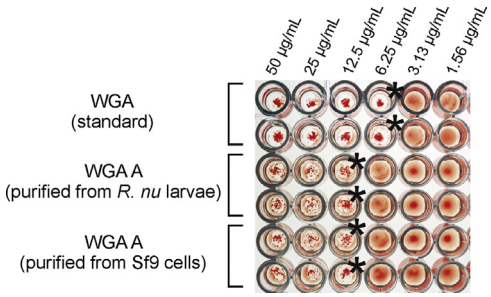

Fig. 8. Agglutination of human red blood cells by commercial wheat germ agglutinin (WGA) and purified recombinant WGA A. Concentrations of commercial WGA and recombinant WGA A were estimated by ELISA. Asterisks indicate the wells corresponding to the minimum WGA concentration giving agglutination.

in diameter) allowed purifying WGA A directly from the upper phase of the aqueous two-phase system, without the need of sample conditioning. The CH250 matrix was inexpensive and easy to manipulate and recovered by simple filtration through a strainer after the steps of adsorption, washing and elution, without any loss of matrix. Also, the eluate of WGA A from the CH250 matrix was clear and not particulate, which would allow further downstream processing using dynamic purification modes.

The SDS-PAGE shown in Fig. 6A shows the complexity of the upper phase from the two-phase aqueous separation system using *R. nu* homogenate expressing WGA A (Fig. 6A, lane 1). The presence of a low amount of lipids and Triton X-114 interfered with the correct resolution of the bands in SDS-PAGE (Fig. 6A, lanes 1 and 2). The identity of the purified WGA A was confirmed by Western blot (Fig. 6B) and RP-HPLC (Fig. 7). The RP-HPLC patterns showed the same time of retention of WGA A (Fig. 7, panel C) and commercial WGA purified from wheat germ (Fig. 7, panel D). The commercial WGA is composed by the three isolectins, A, B and D, of which A is the most abundant. In addition, recombinant WGA A purified from *R. nu* larvae or Sf9 cells was able to agglutinate human red blood cells (Fig. 8). These results demonstrate that the expression of WGA A using BEVS rendered a functional and homogeneous recombinant protein. Each monomer was correctly folded with its 16 disulfide bridges and both formed an active homodimer able to recognize specific sugar moieties (GlcNAc and Neu5Ac) that were present in ovoalbumin and in human red blood cells.

The scale-up of WGA A production might be possible using *R. nu* larvae and the development of an integrated purification method might decrease the overall costs of the WGA purification process. Furthermore, these strategies are environmentally friendly, avoiding the use of large volumes of organic solvent and, in addition, the use of wheat germ, an edible source.

4. Conclusions

WGA is an interesting lectin due to its various applications. Herein, a recombinant WGA A was expressed using BEVS in Sf9 cells and *R. nu* larvae. This recombinant WGA A showed the same behavior as that of commercial WGA. An integrated purification method based on aqueous two-phase separation coupled to affinity chromatography using chitosan mini-spheres might make possible the scaling-up of recombinant WGA A production using *R. nu* larvae as biofactories at low-cost.

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