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High-yield expression of recombinant soybean agglutinin in plants using transient and stable systems

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Abstract Soybean agglutinin (SBA) is a specific
N-acetylgalactosamine-binding plant lectin that can
agglutinate a wide variety of cells. SBA has great
potential for medical and biotechnology-focused appli-
cations, including screening and treatment of breast
cancer, isolation of fetal cells from maternal blood for
genetic screening, the possibility as a carrier system for
oral drug delivery, and utilization as an affinity tag for
high-quality purification of tagged proteins. The success
of these applications, to a large degree, critically
depends on the development of a highly efficient
expression system for a source of recombinant SBA
(rSBA). Here, we demonstrate the utility of transient and
stable expression systems in *Nicotiana benthamiana*
and potato, respectively, for the production of rSBA,
with the transgenic protein accumulated to 4% of total
soluble protein (TSP) in *Nicotiana benthamiana* leaves
and 0.3% of TSP in potato tubers. Furthermore, we show
that both plant-derived rSBAs retain their ability to
induce the agglutination of red blood cells, are similarly
glycosylated when compared with native SBA, retained
their binding specificity for *N*-acetylgalactosamine, and
were highly resistant to degradation in simulated gastric
and intestinal fluids. Affinity column purification using
N-acetylgalactosamine as a specific ligand resulted in
high recovery and purity of rSBA. This work is the first
step toward use of rSBA for various new applications,
including the development of rSBA as a novel affinity
tag for simplified purification of tagged proteins and as a
new carrier molecule for delivery of oral drugs.

Keywords Soybean agglutinin · *Nicotiana
benthamiana* · *Solanum tuberosum* ·
Hemagglutination · *N*-Acetylgalactosamine ·
Protein purification

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Introduction

Soybean agglutinin (SBA) is a legume lectin
glycoprotein that binds non-covalently to specific

cell-surface carbohydrates, provoking agglutination of the bound cells when in solution. SBA has been used to fractionate different cell types for use in clinical and biomedical applications. One such application is the separation of pluripotent stem cells from human bone marrow. Cells fractionated by SBA do not produce graft versus host disease (GVHD) and can be used in bone marrow transplantation across histocompatibility barriers (Yura et al. 2008). Another application is the enrichment and isolation of fetal cells from the blood of pregnant women as a means of detecting fetal genetic and chromosomal abnormalities. It has been shown that the number of fetal erythroblasts recovered by a soybean agglutinin galactose-specific lectin method was approximately eightfold higher than the number obtained by a standardized magnetic cell-sorting (MACS) procedure (Babochkina et al. 2005). In addition, SBA binds very effectively to some tumor cells and has been used to detect and treat several cancers including breast cancer (Pusztai et al. 2008). SBA and other lectins, which are carbohydrate-binding proteins that bind sugar residues reversibly and specifically, have also been exploited as a ligand for targeted oral drug delivery, because their glycoprotein and glycolipid targets are integral parts of the enterocyte membrane (Smart 2004). Procedures for high recovery and purity of SBA have been developed, with extraction of SBA from soybean flour resulting in high-quality purification with over 90% yield (Percin et al. 2009), opening the door for the use of recombinant SBA as a potential novel affinity tag for purification of genetically fused proteins. The potential production of a variety of recombinant proteins genetically fused to SBA, however, necessitates the generation of an efficient recombinant production system.

Native SBA is produced in the developing seeds of *Glycine max*, with maximum production in seeds reaching over 2% TSP (Lindstrom et al. 1990). It is relatively simple to obtain large amounts of purified native SBA given that soybean seeds express high levels of native SBA together with the establishment of efficient purification methods. However, many potential applications of SBA in biotechnology and medicine could not be achieved or are difficult to achieve by use of unmodified native SBA. For example, it is difficult to use native SBA as an affinity tag for protein purification unless the target protein is genetically fused to SBA and produced as a recombinant fusion protein. SBA has previously been expressed in transgenic tobacco seeds in order to study the upstream and downstream cis-regulatory sequences mediating the expression of native SBA (Lindstrom et al. 1990). Although a protein band with a molecular mass expected for the monomer of SBA protein was seen on western blot, no analysis or characterization of the recombinant protein itself was performed; it was merely used as a marker for gene expression. Others have successfully expressed SBA in both *E. coli* and monkey BS-C-1 cells in an attempt to generate recombinant SBA in order to analyze the relationship between glycosylation and tetramer assembly (Adar et al. 1997). They demonstrated that the recombinant SBA retained its agglutination ability and specificity for *N*-acetylgalactosamine. The SBA generated in bacteria, however, lacks glycosylation that, while not required for agglutination or assembly, is important to the stability of the bioactive tetramer (Sinha and Surolia 2005). Production in BS-C-1 cells resulted in a similarly glycosylated protein to native SBA with an identical sugar binding profile but for some unknown reason had a lower agglutination ability. The minimum amount of BS-C-1 cell-derived SBA required to induce hemagglutination is 10–20 μg/mL, which is 4–5 times larger than that of native SBA. In addition, for production of a therapeutic protein, animal cells carry an inherent risk of pathogen contamination and have a high production cost due in large part to media costs. Therefore, both bacteria and mammalian cells are not ideal bioreactors for SBA production for therapeutic uses.

Plants are a suitable alternative expression system for recombinant SBA production. As bioreactors, plants enable unlimited scalability, elimination of product contamination by mammalian pathogens, and reduced production costs compared with microbial or animal cell-based systems (Boehm 2007; Ma et al. 2008; Tremblay et al. 2010). Because of their eukaryotic nature, plants can perform the complex post-translational modification and processing required by many transgenic therapeutic proteins for biological and/or immunological function. Furthermore, plant bioreactors have the short turn-around time needed to obtain gram quantities of a recombinant protein in a matter of weeks when expressed transiently. This is not only economically advantageous, but is also critical to meeting challenges

related to quick access to life-saving biotechnology drugs and therapies. In addition, stable edible transgenic plant tissue might enable direct oral delivery of plant-derived therapeutic proteins and peptides, eliminating the need for expensive downstream protein purification and processing.

Our long-term objective is to generate recombinant SBA for use as a carrier molecule for oral delivery of protein and peptide drugs and as an affinity tag for simplified and high-yield, high purity isolation of recombinant proteins. As a first step toward this objective, we report here the transient production of recombinant SBA (rSBA) in *Nicotiana benthamiana* (Nb), a close relative of tobacco (*Nicotiana tabacum*), and stable production in *Solanum tuberosum* (St) under the control of a ubiquitous CaMV35S promoter. We demonstrate transient expression of SBA in Nb plants at levels as high as 4% of total soluble protein (TSP), whereas its stable expression in St tubers reaches 0.3% TSP. Furthermore, NbrSBA and StrSBA are similarly glycosylated compared with native SBA, retain their ability to induce hemagglutination, bind specifically to *N*-acetylgalactosamine, are stable in simulated gastric fluid (SGF) containing pepsin at acidic pH and are rapidly isolated in high purity from total soluble protein.

Materials and methods

Plasmid construction

The cDNA of soybean agglutinin (SBA) was cloned from *Glycine max* cDNA derived from 1 to 5 mm developing seeds. In brief, total messenger RNA was extracted from the seeds using the RNeasy Plant Mini Kit (Qiagen #74903) and converted to cDNA by following the Superscript II procedure (Invitrogen #18064). The primers F 5′ AAT**CCATGG**CTACTTC AAAGTTGAAAACC 3′ and R 5′ **TCTAGA**TTAA TGATGATGATGATGATGGATGGCCTCATGCA ACACA*AAACTT*G 3′ (with addition of a 6XHis-tag to the C-terminus underlined, restriction sites in bold, and a silent mutation to remove an internal HindIII site italicized) were constructed using the previously published sequence for SBA (Genbank Accession #K00821.1). The generated SBA cDNA was then inserted into pUC-19. After confirmation by sequencing, the SBA cDNA was inserted into pRTL-2 via

digestion with NcoI and XbaI, replacing the GUS gene, providing a 35S promoter, 5′ and 3′ UTR (Carrington and Freed 1990). The resulting expression cassette was digested with HindIII and inserted into pBI-101 to create pBI-rSBA. Tri-parental mating was used to transfer pBI-rSBA to *Agrobacterium tumifaciens* strain LBA4404 and confirmed via PCR using specific primers.

Transient expression of SBA in *N. benthamiana*

Transient expression of pBI-rSBA was accomplished by infection of 6–8-week-old leaves of *Nicotiana benthamiana* as described by Sparkes et al. (2006). Briefly, overnight cultures of Agrobacterium were grown until density reached 0.5–1.0 A_{600}, at which time the cells were centrifuged at $800g$ for 10 min, rinsed four times with infiltration medium (0.5% D-glucose, 50 mM MES, 2 mM Na_3PO_4, 0.0001 M acetosyringone), and then resuspended to the desired cell density, between 0.01 to 0.75 A_{600}. The cells were then infiltrated into the abaxial side of the leaves using a 1-mL syringe and infected tissue was harvested each morning at day 1 through 9 post-infection. For co-infiltration with a second Agrobacterium harboring the T-DNA vector encoding the p19 suppressor gene of tomato bushy stunt virus (Lakatos et al. 2004), the two agrobacterial cultures were mixed at equal concentration before infiltration.

Stable expression of SBA in *S. tuberosum*

Solanum tuberosum mini-tubers were generated as described previously (Bourque et al. 1987). Overnight cultures of *Agrobacterium* with pBI-rSBA were used to transform tubers as previously described (Ma et al. 2005). Regenerating plantlets were transferred to magenta boxes containing MS media supplemented with 50 μg/L kanamycin. The presence of the transgene in transgenic plants was confirmed by PCR, and PCR-positive plants were then selected to produce mini tubers for protein expression analysis.

For mini tuber induction, a stem section (1 cm) with one resting auxiliary bud and one fully developed leaf was excised from a sterile magenta-grown transgenic plant. The leaf was removed and the stem was transferred to tuber-inducing medium as described above and placed in darkness at 20°C.

After 4 weeks, the tubers formed (3 mm in diameter) were harvested and used for protein analysis.

Accumulation of rSBA in *Nicotiana benthamiana* and potato plants

Total soluble protein was extracted from infected *N. benthamiana* leaf tissue or potato tuber and then quantified as described previously (Tremblay et al. 2008). Briefly, approximately 100 mg plant material, either *Solanum tuberosum* tuber or *Nicotiana benthamiana* leaf, was ground in a 1.5-mL tube with a plastic pestle and mixed with protein extraction buffer (200 mM Tris pH 8.0, 100 mM NaCl, 400 mM Sucrose, 1 mM phenylmethylsulfonyl fluoride, 10 mM EDTA, 14 mM β-mercaptoethanol, 0.05% Tween-20, 2 µg/mL leupeptin, 2 µg/mL aprotinin). The mixture was incubated on ice for 30 min and clarified by centrifugation at top speed for 10 min at 4°C. The supernatant was transferred to a new 1.5-mL tube and used for all subsequent assays. Protein samples, boiled or unboiled, were loaded on to a 12.5% SDS–PAGE gel and resolved. Gels were then either stained with Coomassie blue to visualize the protein profile or transferred to a PVDF membrane as described previously (Tremblay et al. 2008). The blot was then blocked for 1 h at room temperature with 5% *w/v* milk in TBS-T and washed 3 times, each time for 5 min, with TBS-T. The blots were incubated overnight at 4°C in 1:2000 rabbit anti-SBA (Cedarlane AL-1301-2) in 1/3 blocking buffer 2/3 wash buffer. The blots were washed 3 times, each time for 10 min, in wash buffer and incubated in 1:5,000 goat anti-rabbit-HRP (G-7641, Sigma) for 1 h at room temperature. The blots were washed 3 times, each time for 10 min, in wash buffer and then incubated with SuperSignal West Pico Chemiluminescent Substrate (34080, Pierce, Rockford, IL, USA). Blots were exposed and then developed using a film processor.

Quantification of plant-derived rSBA

The amount of accumulated rSBA in *Nicotiana benthamiana* leaves or *Solanum tuberosum* tubers was quantified by enzyme linked immunosorbent assay (ELISA). Briefly, commercial native SBA standard (Sigma L-1395) and triplicate NtrSBA or StrSBA samples were bound to 96 well plates by

incubation in phosphate buffer overnight at 4°C. The plates were then washed with PBS-T and blocked with 3% BSA in PBS-T for 1 h at room temperature. The plates were washed 3 times and then incubated overnight at 4°C in 1:2,000 rabbit anti-SBA in 1/2 blocking:1/2 wash buffer. The plates were washed 3 times and incubated for 1 h at room temperature with 1:5,000 goat anti-rabbit-HRP in 1/2 blocking:1/2 wash buffer and then rinsed 3 times. The plates were incubated with Substrate Reagent Pack (DY999, R&D Systems) according to the manufacturer's instructions. The color was developed for 20 min and then stopped by addition of an equal volume of 2 M H_2SO_4. The plate was then read using a Thermomax microplate reader (Molecular Devices, USA). Standard curves were calculated and used to determine protein concentrations of the individual samples. Negative control was protein extract prepared from wild-type plant tissue.

Purification of plant-derived rSBA

Purification of rSBA from *Nicotiana benthamiana* or potato tubers was carried out according to the procedure for GE Healthcare HisTrap HP column (#17-5248-01) or using an *N*-acetylgalactosamine–agarose column. For purification with the *N*-acetylgalactosamine–agarose column, agarose columns with pre-bound *N*-acetylgalactosamine (Sigma A2787-5 mL) were washed and equilibrated with 0.1 M NaCl. The TSP containing rSBA was then applied to the column and rinsed with excess 0.1 M NaCl. Samples were taken throughout rinsing and total protein in rinse was determined by spectrophotometry at A_{280}. Once protein levels were negligible, rSBA was eluted with 0.5 M galactose/0.1 M NaCl. The purified protein was confirmed via western blot and then desalted via dialysis against excess 0.5× PBS buffer.

Hemagglutination assay

Hemagglutination assay was performed using 2% rabbit red blood cells (RBC) suspended in saline buffer. Commercial native SBA standard and purified NtrSBA and StrSBA were used for the assay. The proteins were diluted to different concentrations with saline in order to determine the effective unit for each sample, with one unit defined as the amount of SBA

required to induce agglutination (Lin et al. 2008). All agglutination assays were repeated in triplicate. RBC (2%, 50 μL) was added to round-bottomed 96-well plates and then mixed with 50 μL protein dilutions. The mixture was then allowed to settle for 1 h and the results were recorded.

Competitive sugar-binding assay

Sugar-binding assay was carried out as described above for hemagglutination, with the following modifications. One unit of native SBA or NbrSBA or StrSBA was mixed with saline containing 40 μM or 400 μM concentrations of one of the sugars N-acetylgalactosamine, N-acetylglucosamine, arabinose, lactose, or raffinose. The mixture (50 μL) was then added to 50 μL 2% RBC and incubated for 1 h. The results were then recorded as positive or negative for hemagglutination.

Deglycosylation of plant-derived rSBA

Deglycosylation of NbrSBA and StrSBA was carried out with PNGase F according to the manufacturer's procedure (Sigma P-9120-1SET). The samples were then loaded on to an SDS–PAGE gel followed by western blot analysis using anti-SBA antibody. N-linked glycan removal was confirmed via a band shift on the western blot. To confirm the presence of mannose-type glycans on plant-derived rSBA, blots containing both PNGase F-treated and untreated samples were incubated with 20 μg/mL concanavalin A (C-2010, Sigma) at room temperature for 1 h. After several washes with 0.5% Tween-20 in PBS (pH 7.5), the blot was incubated with HRP-conjugated anti-ConA antibodies (HAL-1104-1, E.Y. Laboratories) at 1,000-fold dilution for 1 h. After the same wash step, the blot was incubated with SuperSignal West Pico Chemiluminescent Substrate. The signals were then developed using a film processor.

Digestion of rSBA in SGF and SIF

Analysis of in-vitro digestion of NbrSBA was carried out using either simulated gastric fluid (SGF) or simulated intestinal fluid (SIF). For SGF (0.2 g NaCl, 0.32 g pepsin, 700 μL HCl, in 100 mL H_2O, final pH 2.5) and SIF (0.68 g monobasic KH_2PO_4, 7.7 mL 0.2 M NaOH, 1.0 g pancreatin, in 100 mL H_2O, final

pH ~6.8), purified protein was incubated in a 37°C water bath with samples taken and mixed in neutralization buffer (1.7 g Na_2CO_3 in 100 mL H_2O) at time 0, 15 s, 30 s, 1 min, 5 min, 15 min, and 30 min. Samples were boiled for 10 min, separated on a 12.5% SDS–PAGE gel and subjected to western blot analysis as described above. Control native SBA and a non-glycoprotein, human GAD65 made in E. coli (Plantigen), were also tested.

Results

Isolation and cloning of cDNA encoding SBA

To obtain a cDNA clone encoding SBA, RNA was extracted from wild-type soybean seeds that were approximately 1–5 mm in size, then converted into cDNA that was then used as a template for the PCR cloning of the SBA coding sequence. The primers included the addition of a 5′ NcoI and 3′XbaI restriction sites to facilitate sub-cloning of the PCR products, and addition of the 6× histidine tag to the C-terminus. The cloned SBA gene was confirmed by sequencing. For the convenience of sub-cloning, the internal HindIII site within the native SBA coding sequence was removed by converting a G to an A in the 2nd codon position encoding serine, resulting in no change of the amino acid. The complete SBA expression cassette was released from the pRTL-rSBA via HindIII digestion and inserted into pBI101.1 to form pBI-rSBA (Supplemental Fig. 1). The plasmid pBI-rSBA was then transferred into A. tumifaciens strain LBA4404 for plant transformations.

Transient production of rSBA in Nicotiana benthamiana

Six-week-old N. benthamiana plants were infiltrated with the A. tumefaciens clone harboring pBI-rSBA or mixed with cultures of A. tumefaciens containing the p19 expression cassette, a viral inhibitor of the RNA silencing pathway in plants that has proven effective in increasing the yield of transiently expressed proteins (Lakatos et al. 2004) (Fig. 1a). Leaf samples were collected from three independent plants on different days post-infiltration with different concentrations of A. tumefaciens cultures, with an equal amount of p19 added for each assay, in order to determine the peak of
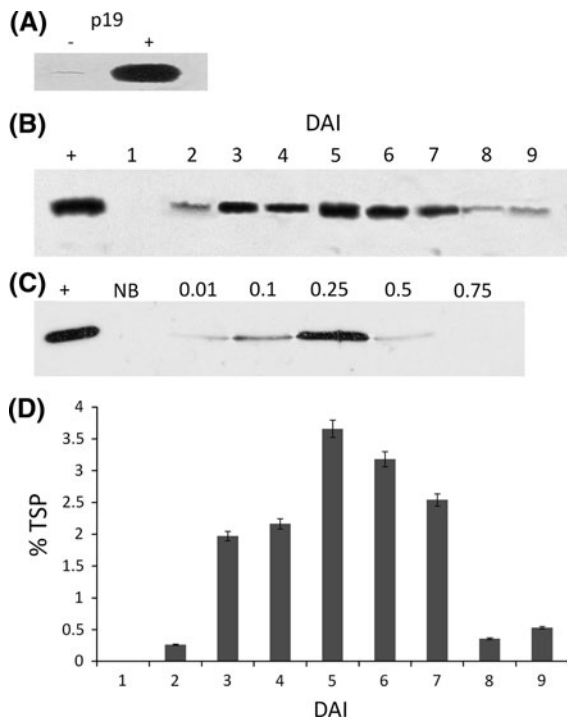
Fig. 1 Western blot analysis of transient protein expression of rSBA in *Nicotiana benthamiana*. **a** Western blot showing the accumulation of NbrSBA with (+) and without (−) p19, which was used in all subsequent transient assays. **b** Accumulation of rSBA from 1 to 9 days after infection (*DAI*). + is native SBA. **c** Accumulation of rSBA at day 5 with different A_{600} concentrations of *Agrobacterium*. + is native SBA, NB is *Nicotiana benthamiana* wild-type TSP. **d** ELISA data for accumulation at days 1–9 after infection at optimized concentration. Values are expressed as a percentage of total soluble protein (*TSP*), with each *bar* representing the mean value of the three collected samples repeated in triplicate from each day, with standard error. All western blots were loaded with approximately 30 μg TSP loaded per lane, 150 ng native SBA control

rSBA expression in the agroinfiltrated *N. benthamiana* plants. The results of immunoblot analysis showed that the highest expression using the gene-silencing suppressor p19 was reached at day 5, with an optimum concentration of *Agrobacterium* of 0.25 A_{600} (Fig. 1b,c). As calculated by ELISA, the expression level of rSBA at day 5 reached 4% of TSP (Fig. 1d). Examination of unboiled samples on SDS–PAGE gels followed by western blotting showed the presence of two bands, one corresponding in size to the monomer and the other equivalent to the size of the tetramer of SBA (Supplemental Fig. 2), suggesting that *N. benthamiana* made rSBA was assembled into a tetrameric protein complex, essential for its biological activity.

Production of rSBA in stable *Solanum tuberosum*

Stably transformed potato plants were also tested for expression of rSBA. Thirteen individual transgenic potato lines were generated after Agrobacterium-mediated transformation and transferred to mini-tuber-inducing magenta boxes in order to generate consistently sized tubers for further analysis. Immunoblot analysis of rSBA expression in unboiled samples of tuber extract showed two major bands of 32 kDa and just over 100 kDa (Supplemental Fig. 2), expected for the monomer and tetramer of SBA, respectively. Immunoblot analysis of boiled samples of tuber extract showed only the small band of 32 kDa (data not shown). The level of StrSBA accumulation in potato mini-tubers was variable among individual transgenic lines, ranging from no detectable signal to 0.31% TSP (Supplemental Fig. 3).

Purification of plant-derived rSBA

Soybean agglutinin (SBA) specifically binds to *N*-acetylgalactosamine, enabling the purification of this protein via an *N*-acetylgalactosamine–agarose column to a high degree of purity. Purification through this column also serves as confirmation of authentic SBA production. Figure 2a shows that sugar-specific purification of rSBA from *Nicotiana benthamiana* leaf extracts resulted in efficient protein retention during purification and elution. In addition, the sugar-purified sample was of high-quality when examined on SDS–PAGE gel and Coomassie blue staining, with negligible contamination of non-specific proteins (Fig. 2b). Sugar-specific purification of rSBA from potato mini tubers also resulted in high-quality, purified protein (data not shown). As a control, the isolation of *Nicotiana benthamiana*-derived rSBA using a traditional HisTrap column was also performed (Fig. 2b).

Hemagglutinating activity of plant-derived r*SBA*

Soybean agglutinin is known to agglutinate human and animal red blood cells (hemagglutination). rSBA was therefore assessed for its ability to agglutinate rabbit erythrocytes. Purified NbrSBA was found to
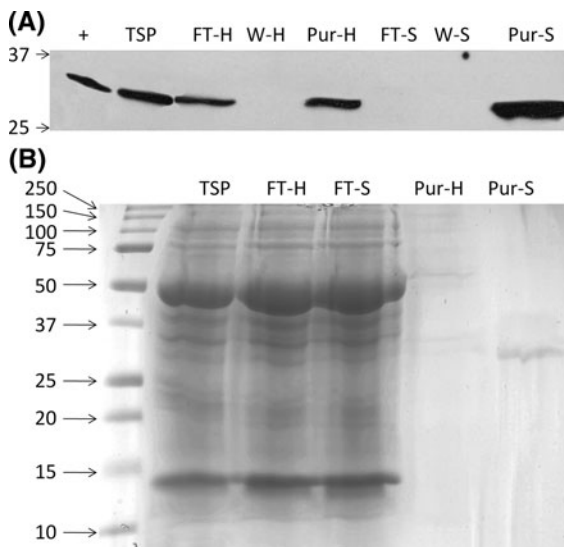
Fig. 2 Purification of rSBA. **a** western blot showing the purification of NbrSBA using either His purification (*H*) or *N*-acetylgalactosamine (*S*). + is native SBA, *TSP* is total soluble protein prepared from *Nicotiana benthamiana*; *FT*, flow through; *W*, wash; *Pur*, purified. No signals were detected in FT-S. **b** Coomassie stained SDS–PAGE gel. Note the single band in Pur-S at 32 kDa, equivalent to the expected monomer size. *Numbers* on the *left* of the blots represent the sizes (kDa) of the protein ladder

induce the agglutination (clumping) of rabbit erythrocytes within 1 h of treatment (Fig. 3a). Similar results were obtained with StrSBA (data not shown). The minimum amount of native SBA required to induce agglutination in the assay was 2.5 μg, whereas NbrSBA and StrSBA required approximately 2 and 3.5 μg, respectively. As agglutination of erythrocytes by SBA is because of its ability to bind to specific sugar residues on the cell surface, it is therefore speculated that the agglutinating activity of SBA could be inhibited by addition of specific sugars. Indeed, addition of *N*-acetylgalactosamine even at low concentrations was able to prevent the agglutinating ability of NbrSBA, similarly to the native SBA control (Fig. 3b). In contrast, the agglutinating ability of NbrSBA or native SBA was unaffected by high concentrations of non-specific sugars (Supplemental Table 1; see also Fig. 3b).

Plant-derived rSBA is glycosylated

Native SBA is a glycosylated protein with a single high-mannose glycan (Adar et al. 1997). The

presence of glycosylation, while not required for assembly or function, is important in maintaining the stability of SBA (Sinha and Surolia 2007). To determine the glycosylation status of plant-derived rSBA, NbrSBA, StrSBA, and SBA standard were digested with PNGase F and analyzed by immunoblotting following separation by SDS–PAGE gels. Both SBA standard and plant-derived rSBA had a decrease in molecular size of approximately 2–3 kDa, which is an expected size for a single glycan (Fig. 4a). To determine the type of glycans of plant-derived rSBA, blots containing both PNGase F–treated and untreated NbrSBA, StrSBA, and SBA standard were incubated with ConA, which binds to *N*-linked mannose, and then with anti-ConA antibody. Figure 4b shows that binding of ConA occurs only in the untreated samples, with no binding to any of the PNGase F treated SBA proteins, suggesting that plant-derived rSBA contains high-mannose type glycans, similar to native SBA.

Plant-derived rSBA is stable in simulated gastric and intestinal fluids

Plant-derived rSBA was further investigated in simulated gastric and simulated intestinal fluid (SGF and SIF) to assess its stability in the digestive system. SGF (3.2 g/L pepsin, pH 2.5) has been used to mimic the acidic stomach environment in animals, and SIF (10 g/L pancreatin, pH 6.8) is used for proximal small bowel conditions. To this end, purified NbrSBA and native SBA were used in simulated digestion experiments. The effect of SGF and SIF on the degradation of NbrSBA, as evaluated by immunoblot analysis, is shown in Fig. 5a,b. As can be seen, NbrSBA remains relatively intact after digestion even for 30 min in either solution. Native SBA showed similar resistance to SGF and SIF digestion (data not shown). To ensure proper activity of the simulated fluids, *E. coli*-derived recombinant GAD65 (glutamic acid decarboxylase) previously produced in our laboratory was tested as a control. The protein lasts less than 1 min in SGF and 5 min in SIF (Fig 5c,d).

Discussion

We have demonstrated the successful recombinant production of SBA through both stable and transient
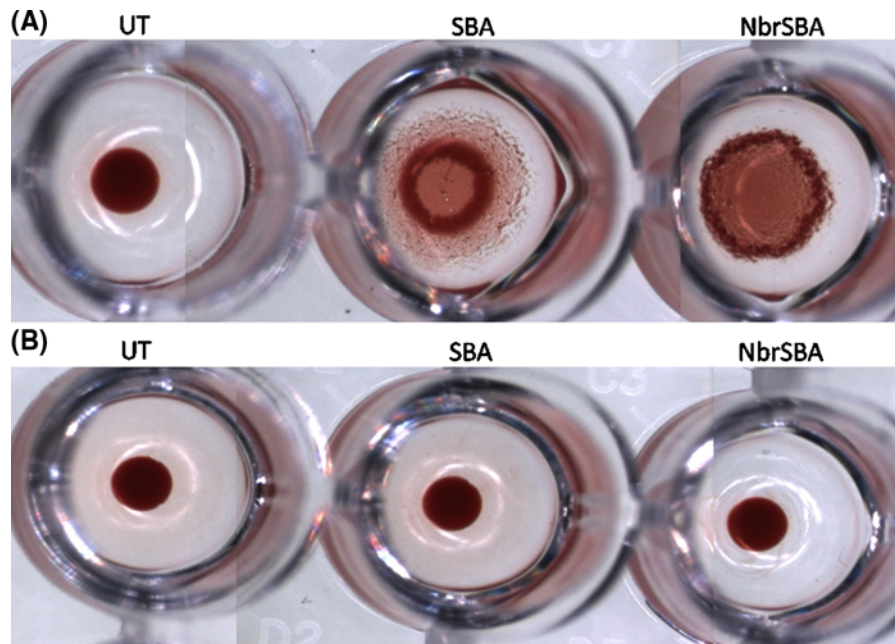
Fig. 3 Hemagglutination/sugar inhibition study. Rabbit red blood cells undergo agglutination when treated with SBA, because of the presence of *N*-acetylgalactosamine on membrane-bound proteins. **a** Agglutination of rabbit red blood cells (RBC) following treatment with native SBA control or *Nicotiana benthamiana*-derived rSBA (*NbrSBA*), resulting in clumping around the bottom of the well. *UT*, untreated RBC, as characterized by RBC settling to the bottom of the well. **b** *N*-acetylgalactosamine inhibition. Both native SBA and *Nicotiana benthamiana*-derived rSBA are inhibited by *N*-acetylgalactosamine, enabling RBCs to pellet

expression in *Solanum tuberosum* and *Nicotiana benthamiana*, respectively. SBA has served as an excellent system of choice to study the fundamentals of protein–carbohydrate interaction (Loris et al. 1998; Sharon and Lis 1990). With a vast increase in knowledge of SBA and other lectins, it has become apparent that SBA has many additional applications, especially in medical and biotechnology-focused industrial areas such as the utilization of SBA to selectively remove cancerous cells from whole blood without removing normal T cells, to prevent graft-versus-host disease following transplant, or to enrich hematopoietic stem cells (Bakalova and Ohba 2003; Kernan et al. 1987; Yura et al. 2008). Moreover, recombinant SBA has the potential to serve as a carrier system for oral drug delivery or to be used as an affinity tag for isolation of high-purity fusion proteins. To make these applications feasible, however, a more affordable source of large quantities of high-quality rSBA is required. Previous recombinant production of SBA in tobacco was incidental, because it was simply used as a marker for studying the promoter activity and, as such, no assays were

performed to confirm that the protein made retained authentic SBA activity (Lindstrom et al. 1990). Similarly, recombinant production in both bacterial and mammalian cell culture was for the purpose of elucidating the functional role of glycosylation and the produced recombinant SBA was either lacking glycosylation (*E. coli*-derived) or had reduced agglutinating ability (mammalian cell-derived) (Adar et al. 1997). Our objective was to develop a robust expression system that can generate large quantities of low-cost authentic rSBA, which retains its binding specificity to facilitate high-purity simplified protein isolation and maintains its stability in the gastrointestinal tract (GI) needed for future use as a carrier molecule for oral drug delivery.

Authenticity of both NbrSBA and StrSBA was confirmed by several results. The foremost is the binding and inhibition studies using rabbit red blood cells. SBA binds to different surface proteins bearing *N*-acetylgalactosamine, and, in the case of red blood cells, results in hemagglutination (Gordon and Marquard 1974). Both of the plant-derived rSBAs were able to induce hemagglutination of rabbit red
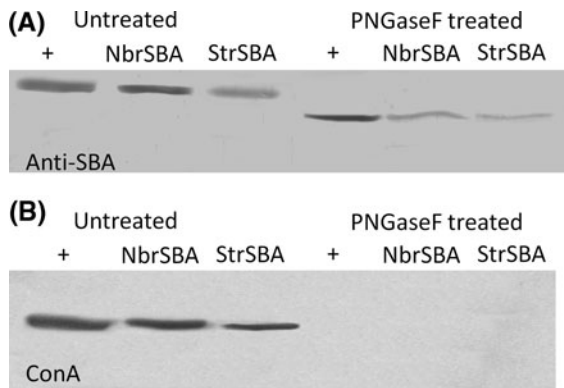
(A) Untreated — PNGaseF treated
+ NbrSBA StrSBA + NbrSBA StrSBA

Anti-SBA

(B) Untreated — PNGaseF treated
+ NbrSBA StrSBA + NbrSBA StrSBA

ConA

Fig. 4 Determination of glycosylation of plant-derived rSBA. **a** Deglycosylation of NbrSBA and StrSBA using PNGase F, which cleaves *N*-linked glycans. Native SBA (+), NbrSBA, and StrSBA were treated by PNGase F, and analyzed by SDS–PAGE and western blotting. Untreated samples were used as controls. + is native SBA. **b** Concanavalin A binding analysis. Plant-derived rSBA and native SBA, both untreated and treated with PNGase F, were treated with ConA, which binds to *N*-linked mannose. ConA-treated samples were analyzed by SDS–PAGE and western blotting using mouse anti-ConA antibody. Binding to both untreated native and recombinant SBA, and no binding to PNGase F treated samples, suggests the presence of high mannose-type glycans for both recombinant and native SBA

(A) NbrSBA in SGF
0 15s 30s 1m 5m 15m 30m

(B) NbrSBA in SIF
0 15s 30s 1m 5m 15m 30m

(C) hGAD65 in SGF
0 15s 30s 1m 5m 15m 30m

(D) hGAD65 in SIF
0 15s 30s 1m 5m 15m 30m

Fig. 5 In-vitro digestion of *Nicotiana benthamiana*-derived rSBA in SGF and SIF. **a** NtrSBA is not degraded in simulated gastric fluid (*SGF*), even after incubation for 30 min (*m*), demonstrating resistance to both low pH and pepsin digestion. **b** NtrSBA is stable during simulated intestinal fluid (*SIF*) digestion. **c** and **d** *E. coli*-made hGAD65 is rapidly degraded in SGF and SIF, respectively. Each *lane* contains the starting equivalent of ~500 ng purified protein

blood cells, as did commercial native SBA standard. In addition, a similar minimum amount of SBA, whether native or from *N. benthamiana* or *S. tuberosum*, was able to induce agglutination. This suggests that, unlike monkey cell-made SBA, Nb/StrSBA is functionally equivalent to native SBA. Many lectins can induce hemagglutination, however, so it is important to confirm the specificity of rSBA for its ligand. For example, the Chinese black soybean variety is able to cause agglutination of rabbit red blood cells but is inhibited by a different sugar, in that case melibiose (Lin et al. 2008). The inhibition of hemagglutination by addition of *N*-acetylgalactosamine but not other sugars confirms the rSBA retains the authentic binding profile of native SBA. In addition to this, the ability to specifically purify high-quality rSBA via *N*-acetylgalactosamine bound to agarose further confirms the authenticity of the recombinant protein, as there was no detectable level of rSBA in either the flow-through or wash steps, only in the elution step. The capture rate for this technique has been reported to be over 90% efficient, which is supported by the lack of rSBA signal in the flow-through steps seen in Fig. 2b (Percin et al. 2009).
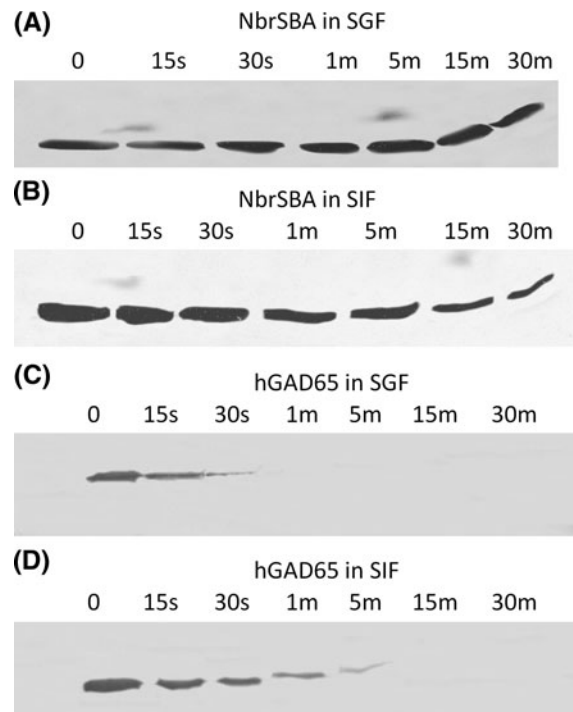
The ability to purify high-quality rSBA also enables potential use as an affinity tag. Through genetic fusion with a gene of interest (GOI), it may be possible to first purify the fusion protein on the sugar columns, followed by subsequent cleavage of the desired GOI via specific endoproteolytic cleavage, such as with tobacco etch virus (TEV) protease (Tubb et al. 2009). A second possibility would be in combination with a linker intein, an intervening protein sequence that can be induced to undergo N-terminal, C-terminal or both termini cleavage under specific conditions (Evans et al. 2005). This could enable cleavage of the GOI from rSBA without any expensive proteases and result in high-purity protein. It should be mentioned that because of the requirement of the tetrameric formation for proper SBA activity, addition of a fusion partner may affect the formation of a tetrameric SBA. However, several groups, including our own, have previously

demonstrated that other multimer forming proteins, for example the non-toxic B subunit of cholera toxin, retain their ability to form pentamers and bind to their target ligand when fused with other proteins (Arakawa et al. 1998; Li et al. 2006; Ruhlman et al. 2007; Tremblay et al. 2008).

The production of rSBA was accomplished using both transient and stable transformation of *Nicotiana benthamiana* and potato, respectively. Transient gene expression in plants has a number of advantages over stable genomic transformation in plants or other recombinant expression platforms. First and foremost is that new recombinant proteins can be generated in sufficient quantities for in-vitro analysis and in-vivo studies at a speed not possible with traditional bioreactors. This is best demonstrated in the generation of idiotpe-specific scFvs to treat non-Hodgkins lymphoma patients. Researchers were able to generate patient-specific scFvs by first cloning the antibody fragment sequences from the patient's biopsy and then generated sufficient quantities in plants to inject the plant-made scFvs back into the patient in less than 16 weeks, something unachievable with any other biological system (McCormick et al. 2008). Transient expression can also be used to generate and validate new vaccines against future pandemics, for example H1N1 or SARS, for which large quantities of vaccine are needed in a short-period of time in order to meet world-wide demand. An additional benefit of transient expression in plants is the level of accumulation. In our work, we achieved almost 4% accumulation of rSBA in less than a week. This is a tenfold increase over the accumulation seen in our stably transformed potato lines. Based on our data, the transient system can generate an approximate yield of between 1.5 and 3 g of rSBA per kg fresh weight in 1 week, meaning that a greenhouse setting capable of producing 1,000 kg of tissue per week could generate up to 3 kg of purified rSBA every week.

The use of stable lines expressing rSBA can likewise prove advantageous. The production of a stable expression platform provides long-term, low-cost therapeutic proteins, typically required where the therapeutic agent is commonly used in multiple individuals over a long period of time, for example in the potential prevention of type 1 diabetes using glutamic acid decarboxylase 65 (GAD65) (Ma et al. 2004). We were able to accumulate rSBA to over 0.3% of TSP in transgenic potato tubers. A major

benefit of tuber accumulation is the stability of recombinant proteins during long periods of storage. In the production of monoclonal antibodies, for example, the recombinant antibodies were stable for over 6 months when stored in the dark at room temperature, with little to no loss in either quantity or activity (De Wilde et al. 2002). This makes potatoes an excellent system for recombinant protein production, because one of the major disadvantages of field/greenhouse grown plants for recombinant production is losses in yield owing to protein degradation during transit and processing. An additional benefit of the use of tubers is that they can be used to orally deliver the target protein with minimal processing, especially given that potato tubers form a staple part of many cultures' diets and can be processed for consumption via freeze-drying that enables ingestion without cooking and thus could help minimize protein denaturation/loss during food preparation.

In-vitro digestion of *N. benthamiana*-derived rSBA under simulated gastric and intestinal conditions proved it to be stable. There was no significant loss or degradation of the protein after incubation of NbrSBA in SGF for 30 min, as seen in Fig. 5a,c. These results suggest that the plant-derived rSBA may hold great promise for use as a carrier system for oral delivery of protein and peptide drugs. The oral route for drug delivery is the most preferred route and has considerable advantages: requiring neither sterile needles nor trained personnel, lower cost and increased quality of life, increased access to a large population, reduced side-effects often seen with systemic delivery, and greater patient compliance and acceptability. However, administration of therapeutic peptide or protein drugs by the oral route is a major challenge. Orally administered peptide or protein drugs are readily degraded because of their exposure to the harsh environment of the GI (low pH and various proteinases and peptidases). Therefore, development of suitable delivery systems is crucial to the success of oral administration of protein drugs. Plant-derived rSBA may provide an ideal vehicle for oral drug delivery. Moreover, SBA undergoes endocytosis through the epithelial lining of the intestine, adding to its potential as an adjuvant for therapeutic protein delivery (Benjamin et al. 1997).

A potential concern with the use of rSBA as a carrier system for targeted drug delivery is its anti-nutritional property. Indeed, SBA is one of the

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predominant anti-nutritional factors found in the raw soybean and accounts for approximately 50% of the growth inhibition in rats fed unheated soybean (Liener 1996). However, its anti-nutritional activity is strictly dose-dependent. A negative effect of SBA on growth and immune function in rats was observed only when very high levels of this protein was given in diets (14 mg/per rat daily or equivalent to 0.2% of its body weight). In our previous work on oral tolerance induction in mice using plant-derived autoantigenic protein, we showed that only microgram quantities (or <0.005% of the animal's body weight) of the plant-derived antigen is required to induce a response (Ma et al. 2004). This suggests that when used as a carrier system, the minimum effective dose of rSBA-antigen fusion protein required to be delivered is in the range of micrograms, not milligrams. Delivery of microgram quantities of SBA to animals is safe (Buttle et al. 2001; Tang et al. 2006). However, as with all therapeutics destined for human use, thorough studies would be required to determine the overall bio-safety.

In conclusion, our work has demonstrated the feasibility of high accumulation, high-purity recombinant production of SBA. This is the first molecular characterization of rSBA in plants. The availability of low-cost, high-quality rSBA may make many important applications of this protein possible, especially in medicine.

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