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Glossina Proteolytic Lectin Does Not Require a Carbohydrate Moiety for Enzymatic or Trypanosome-Transforming Activities

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ABSTRACT The developmental cycle of the cyclically transmitted African trypanosome involves an obligatory passage through the tsetse fly, *Glossina* spp. This intricate relationship requires the presence of molecules within the insect vector, including a midgut lectin, that interact with the trypanosome. Recently, a gene encoding for a proteolytic lectin, with trypanosome-transforming activity, was isolated from a midgut cDNA library of *Glossina fuscipes fuscipes* Austen in our laboratory. Using the same approach, we have identified a similar gene from a midgut cDNA library of *Glossina austeni* (Newstead). The protein encoded by this gene was expressed in bacteria and a baculovirus-based expression system. The baculovirus-expressed lectin was found in the medium of baculovirus-infected Sf-21 cell cultures, indicating that the tsetse fly-derived signal peptide was recognized and cleaved by the Sf-21 cells. The baculovirus-expressed protein also was glycosylated despite the absence of classical *O*-linked and *N*-linked sugar attachment motifs. Both the baculovirus- and bacterium-expressed lectin proteins were shown to agglutinate trypanosomes and rabbit red blood cells in vitro. This agglutination was strongly inhibited by D-glucosamine. D-Glucosamine also inhibited the action of the authentic and recombinant lectins upon the chromogenic substrate Chromozym TRY. Interestingly, both baculovirus- and bacterium-expressed lectins showed no significant differences in terms of these activities, indicating that a sugar moiety is not essential for biological activity. Our results provide an important molecular tool for further characterization of *Glossina* proteolytic lectin.

KEY WORDS tsetse fly, *Glossina*, proteolytic lectin, trypanosome

TSETSE FLIES, *Glossina* spp. (Diptera: Glossinidae), are the only known vectors of African trypanosomiasis, a debilitating disease affecting humans and other vertebrates. These flies are found only in Africa south of the Sahara and have been associated with the transmission of African trypanosomiasis for over a century (Bruce et al. 1909). However, it is only within the past two decades that numerous efforts have made toward understanding the interactions between *Glossina* species and cyclically transmitted African trypanosomes. These efforts have focused on factors within *Glossina* midgut that are principally protein in nature (Otieno et al. 1983, Ibrahim et al. 1984, Maudlin and Welburn 1987). Ibrahim et al. (1984) were the first to show that a crude homogenate of tsetse midgut tissue is able to agglutinate trypanosomes. Subsequently, numerous studies (Maudlin and Welburn 1988a, b; Stiles et al. 1990; Maudlin 1991; Imbuga et al. 1992; Abubakar et al. 1995; Osir et al. 1999) have identified protein components of the tsetse fly midgut tissue that are capable of interacting with trypanosomes, including the agglutination of the bloodstream forms of trypanosomes in vitro. The key molecule involved in this agglutination activity is a bloodmeal-induced lectin (Abubakar et al. 1995) that is inhibited by sugars, especially D-glucosamine (Maudlin and Welburn 1987, Mihok et al. 1992). When fed to tsetse, D-glucosamine strongly increases the establishment of trypanosomes within the midgut.

Osir et al. (1995) have purified an active proteolytic lectin protein from midgut homogenates of *Glossina longipennis* Corti and *Glossina fuscipes fuscipes* Austen (Abubakar et al. 2003). These studies have shown that the proteolytic lectin is indeed the active molecule that transforms the bloodstream form of trypanosomes to a procyclic form (Welburn et al. 1989, Welburn and Maudlin 1999, Abubakar et al. 2003). Knowledge of the inhibitory property of D-glucosamine on the midgut proteolytic lectin has facilitated the understanding of the teneral puzzle, because newly emerged tsetse flies are more susceptible to trypanosome infection than old flies. Another important biochemical property of the purified proteolytic lectin molecule is that it is glycosylated (Osir et al. 1995). The functional significance, if any, of this glycosylation has not been clarified. Recently, a gene that encodes a lectin protein was isolated from a midgut cDNA library of *G. fuscipes*

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fuscipes and expressed in bacteria (Abubakar et al. 2005). Here, we report a comparative study in which a lectin gene from another tsetse fly, *Glossina austeni* (Newstead), was isolated and expressed in bacteria and by a recombinant baculovirus in a lepidopteran cell line. The proteolytic and agglutination activities of the lectin expressed in bacteria were highly similar to those of the authentic and baculovirus-expressed proteins, suggesting that glycosylation is not essential for biological activity.

Materials and Methods

Construction of a Midgut cDNA Expression Library of *G. austeni*. Total RNAs were extracted from the midguts of 50 male *G. austeni* obtained from a laboratory colony maintained at the Animal Rearing and Containment Unit (ARCU) of the International Center of Insect Physiology and Ecology (ICIPE). The teneral (24-h-postemergence) flies were subjected to two rounds of feeding and starvation as described by Abubakar et al. (1995). Essentially, the feeding and starvation regime involved allowing the insects to feed on pig blood for 30 min through an artificial silicone membrane followed by starvation for 72 h. This procedure was then repeated before the removal of the midguts. After removal, the midguts were rinsed three times in Tris buffer (20 mM Tris-Cl, pH 8.0) and homogenized before total RNA extraction using an RNA extraction kit (Promega, Madison, WI). Double-stranded cDNAs were generated from the total RNAs using a SMART cDNA library construction kit (BD Biosciences Clontech, Palo Alto, CA). The cDNAs were then digested with *Sfi*IA and *Sfi*IB, size fractionated, and directionally ligated into pTriplEx2 (BD Biosciences Clontech).

Antibody Screening of the cDNA Library. The *G. austeni* cDNA library was screened following a procedure already established in the laboratory (Abubakar et al. 2005). Briefly, polyclonal antibodies against *Glossina* proteolytic lectin (Gpl) were prepared as described by Osir et al. (1986). Nonspecific binding to bacterial proteins was prevented by preadsorbing the serum with a lysate (diluted 1:10 in 2 ml of 25 mM Tris-Cl, 135 mM NaCl, 2 mM KCl [Tris-buffered saline] pH 7.4) from *Escherichia coli* DH5α cells (protein concentration of 50 μg/ml). The preadsorbed serum containing the polyclonal antibodies was further diluted (1:300), and used for library screening according to the method of Sambrook et al. (1989). After colony transfer, the membranes were stained, and positive clones were visualized with 5-bromo-4-chloro-3-indolyl-phosphate (Sigma, St. Louis, MO) and nitroblue tetrazolium (Sigma) substrates in the dark. The plasmid DNAs in clones that were confirmed to be positive against the anti-Gpl serum were extracted using a Miniprep kit (QIAGEN, Valencia, CA) and their inserts were sequenced.

Database Search and Sequence Analysis. The National Center for Biotechnology Information (Bethesda, MD) BLAST computer search program was used to perform sequence homology searches against public databases (Altschul et al. 1997). Sequence alignment and statistical analysis of alignments were performed using the MultiAlign program (Corpet 1988).

Construction of a Recombinant Baculovirus Expressing Gpl. A polymerase chain reaction (PCR) was used to amplify the complete open reading frame of the *gpl* gene by using the primers Gpl5BglII (5′-GCA GAT CTA TGA AGT TCT TTG CAG TGT TCG C-3′) and Gpl3BglII (5′-GCA GAT CTT TAC AAA AGT TGC GCA TAG TTC-3′) and *Pfu* polymerase (Invitrogen, Carlsbad, CA). Plasmid DNAs isolated from positive clones after antibody screening (see above) were used as template DNAs. The thermo cycling parameters were 1 cycle of 94°C for 1 min; 39 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min; followed by a clean up step of 72°C for 3 min. This amplification incorporated *Bgl*II sites at the 5′ and 3′ ends of the *gpl* gene. After gel purification, the PCR-amplified fragment was first blunt-end ligated into the *Sma*I site of pUC18. Subsequently, the *gpl* gene was excised by *Bgl*II digestion and cloned into the *Bgl*II site of the baculovirus transfer vector pAcUW21 (Weyer et al. 1990). Restriction endonuclease digestion and nucleotide sequencing confirmed the orientation and authenticity of the *gpl* gene in the resulting recombinant baculovirus transfer vector, pAcUW21-gpl.

To generate a recombinant baculovirus expressing the *gpl* gene, pAcUW21-gpl (4 μg) was transfected with linearized, chitinase-negative AcMNPV DNA (0.1 μg, a generous gift from Prof. Linda King, Oxford Brookes University, Oxford, United Kingdom) by using Cellfectin reagent (Invitrogen) basically as described by O'Reilly et al. (1992). After the transfection, a recombinant baculovirus (AcMNPV-gpl) was isolated by two rounds of plaque purification on Sf-21 cells. The authenticity of the construct was confirmed by polymerase chain reaction (PCR) by using AcMNPV- and *gpl*-specific primers. The virus was amplified on Sf-21 cells, and stocks were kept at −70°C and +4°C.

Cell Culture and Expression of *Glossina* Proteolytic Lectin. Routinely, Sf-21 cells were grown at 27°C in TC-100 medium with L-glutamine (JRH Scientific, Lenexa, KS) that was supplemented with 10% fetal calf serum (Intergen, Purchase, NY). For recombinant protein production, Sf-21 cells in suspension (5×10^5 cells/ml) were inoculated at a multiplicity of infection of 0.1 plaque-forming units per cell. Sf-21 cells inoculated with AcMNPV-lacZ under identical conditions were used as a control. At 72 h postinfection, the cells were harvested by centrifugation (2,000 × *g*; 5 min) and then resuspended in 5 ml of saline buffer (BIS; 10 mM Tris-Cl, pH 7.9, 130 mM NaCl, 5 mM KCl, and 1 mM $CaCl_2$). The cells were lysed by sonication, and the homogenate was centrifuged at 4,000 × *g* for 10 min at 4°C. The supernatant, within a dialysis bag (6,000–8,000 mol. wt. cut-off, Millipore Corporation, Billerica, MA) was concentrated by polyethylene glycol precipitation (PEG 40,000, Serva, Heidelberg, Germany) and dialyzed against 6 liters of BIS for 8 h. Protein concentration was estimated using a BCA pro-

tein assay kit (Pierce Chemical, Rockford, IL) with bovine serum albumin as a standard. The mass and purity of the recombinant protein was estimated by sodium dodecyl sulfate gradient-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). Gradient gels were cast using a gradient maker (Invitrogen) and stained by Coomassie brilliant blue and silver staining procedures.

Protein Expression in *E. coli*. Recombinant GPL was expressed in *E. coli* strain BL21 from a pTriplEx2 vector containing the full-length cDNA of Gpl. The transformed bacteria were cultured in Luria-Bertani medium containing 125 μg/ml ampicillin and incubated at 37°C until they reached a log phase of growth. At this point, 5 ml of cells was transferred aseptically to a flask containing 300 ml of TYP broth (16 g of Bacto tryptone, 16 g of yeast extract, 5 g of NaCl, and 2.5 g of K_2HPO_4). Protein expression was induced with isopropyl β-D-thiogalactoside (1 mM final concentration) when the OD_{600} reached 0.6 (≈2 h). The induced cells were allowed to grow overnight (12 h) with shaking at 37°C. The cells were pelleted by centrifugation (3,000 × *g;* 4°C; 10 min) and resuspended in phosphate buffered saline (pH 8.0) and sonicated on ice. Next, the lysate was centrifuged (8,000 × *g;* 20 min; 4°C), and the resultant supernatant dialyzed against 6 liters of BIS for 8 h and stored at −20°C.

Preparation of Trypanosomes and Rabbit Red Blood Cells. *Trypanosoma brucei brucei* ILTat 1.4 (Miller and Turner 1981), obtained from the International Livestock Research Institute (ILRI, Nairobi, Kenya), were passaged in male Wistar rats as described by Imbuga et al. (1992). The rats were obtained from the Animal Rearing and Containment Unit (ARCU) of ICIPE. Parasites (in vivo-derived trypanosomes) were harvested from the rats by cardiac puncture following the method of Lanham and Godfrey (1970) and were purified from the blood by using a DEAE Sephacel column. Additionally, bloodstream trypanosomes (i.e., in vitro-derived trypanosomes) were maintained in vitro following the procedure described by Hirumi and Hirumi (1989).

Purification of Proteolytic Lectin from Midgut Homogenates and Bacterium-Expressed Gpl. Authentic proteolytic lectin was purified from 200 tsetse fly midguts by using a two-step approach as described by Abubakar et al. (2003). Briefly, the first step involved applying ≈90 mg of crude midgut homogenate onto a DEAE Sephacel column equilibrated with 20 mM Tris-Cl (pH 8.0). After washing with the same buffer, the bound proteins were eluted using a 0–0.5 M NaCl gradient in the same buffer. The eluted proteins were first dialyzed against 6 liters of BIS buffer for 24 h and applied onto a D-glucosamine affinity column. The bound protein was eluted from the column with 0.2 M D-glucosamine in BIS buffer. The same procedure was used to purify the recombinant, *E. coli*-expressed Gpl.

Immunoaffinity Purification of Baculovirus-Expressed Gpl. To purify the baculovirus-expressed Gpl, an affinity matrix was generated by coupling polyclonal antibodies that were generated against the recombinant bacteria-expressed GPL to cyanogen bromide-activated Sepharose 4B (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) following the manufacturer's protocol. The affinity column (3.5 ml) was equilibrated with wash buffer (10 mM Tris-Cl, pH 8.0, 0.14 M NaCl, and 0.025% NaN_3). Then, 1.5 ml of the concentrated supernatant of virus-infected cells (see above, containing ≈3 mg of crude protein) was loaded onto the column. The gel was washed first with 50 ml of wash buffer and second with 40 ml of buffer A (50 mM Tris-Cl, pH 8.0, 0.1% Triton X-100, and 0.5 M NaCl). The bound proteins were eluted with 40 ml of buffer B (50 mM Tris-Cl, pH 9.0, 0.1% Triton X-100, 0.5 M NaCl). The flow rate was maintained at 250 μl/min using a peristaltic pump for all of the washes and elution. The absorbance (A_{280}) of the eluate (1 ml per fraction) was measured using a spectrophotometer. The eluted sample was first dialyzed against 6 liters of phosphate-buffered saline (PBS) (pH 8.0) for 12 h, concentrated with polyethylene glycol (PEG 40,000, Serva), and then dialyzed against 6 liters of PBS (pH 8.0) for 8 h.

Agglutination and Trypsinization Assays. The ability of the purified proteins to agglutinate washed trypanosomes and rabbit red blood cells was determined as described by Abubakar et al. (1995). The trypsinization activity of the recombinant Gpls was determined as described previously (Imbuga et al. 1992) with carbo-benzoxy-val-gly-arg-4-nitroanilide (Chromozym-TRY; Roche Diagnostics, Mannheim, Germany).

Periodic Acid Schiff (PAS) Staining for Glycoproteins. SDS-PAGE was carried out using a 12.5% slab gel as described above. After electrophoresis the gel was placed in a fixative [7.5% (vol:vol) acetic acid] and rocked gently for 10 min at room temperature. The gel was then treated with 1% (wt:vol) periodic acid (Sigma) for 15 min at 4°C to oxidize the oligosaccharides. Subsequently, the gel was rinsed with distilled water for 30 min at room temperature and stained with Schiff's reagent (Sigma) in the dark (15 min; 4°C). After staining, the gel was treated with 0.5% (wt:vol) sodium metabisulfite (Sigma) for 30 min at room temperature. Finally, the gel was rinsed extensively with distilled water at room temperature.

Results

Cloning and Expression of *G. austeni* Proteolytic Lectin. Four Gpl-positive clones (≈0.02%) were detected from five filters containing ≈4,500 colonies. The clones were purified to homogeneity and their cDNA inserts were sequenced. The sequences of all of the inserts were identical. The cDNA sequence of the proteolytic lectin from *G. austeni* is shown in Fig. 1A (GenBank accession no. DQ060150). The cDNA contained an open reading frame (Fig. 1A, nucleotides 30–852) that encoded a predicted protein of 274 amino acid residues. The calculated molecular mass of the deduced protein was 29 kDa. A putative signal peptide (amino acid residues 1–17) was identified. Sequence motifs conserved in serine proteases also

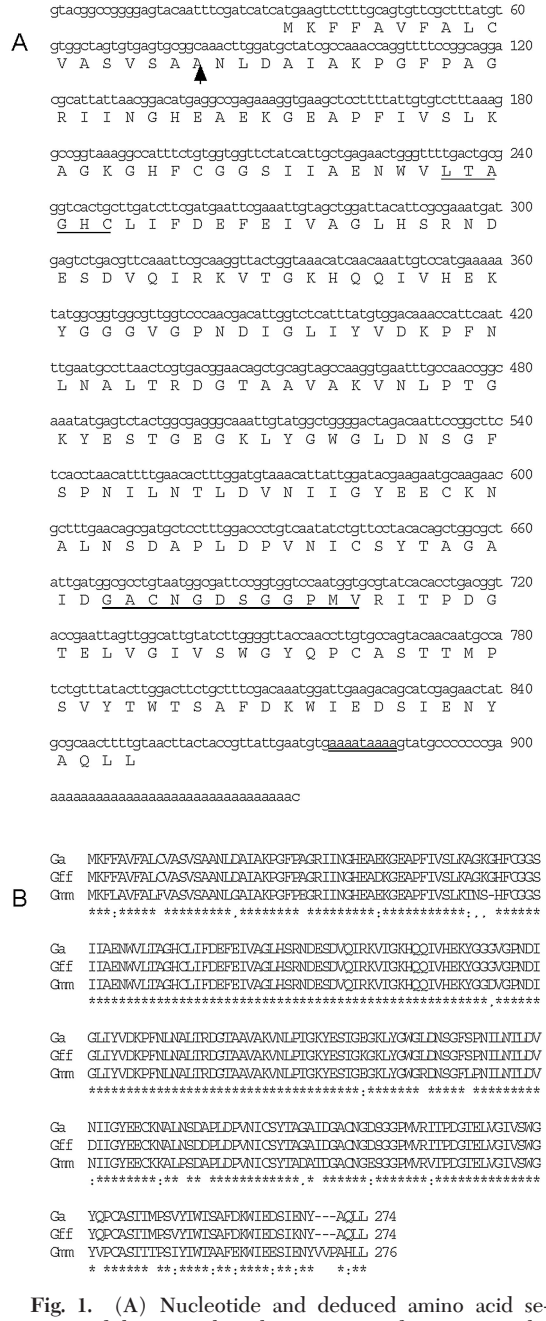

Fig. 1. (A) Nucleotide and deduced amino acid sequences of the proteolytic lectin cDNA of *G. austeni*. The arrowhead indicates the predicted signal peptide cleavage site. The putative serine protease active site residues are underlined. A potential polyadenylation signal is double underlined. (B) Alignment of the deduced protein sequences of proteolytic lectins from *G. austeni* (Ga) and *G. fuscipes fuscipes* (Gff), and a chymotrypsin-like serine protease from *G. morsitans morsitans* (Grm). Identical (*) and similar (:) amino acids residues are indicated below.

were found between amino acid residues 68–73 and 213–224 (underlined in Fig. 1A). The deduced protein sequence showed 98% identity to a Gpl that was previously cloned from *G. fuscipes fuscipes* midguts (Abubakar et al. 2005) and 91% identity to a chymotrypsin-like serine protease from *G. morsitans morsitans* (Yan et al. 2001) (Fig. 1B).

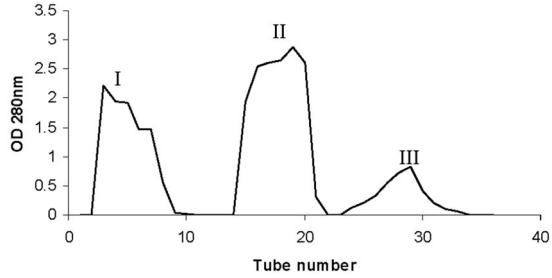

Fig. 2. Immuno affinity chromatographic purification of recombinant Gpl expressed in Sf21 cell lines. The glucosamine-affinity column was loaded with the culture medium of AcMNPV-gpl-infected Sf-21 cells. After elution of unbound proteins (peak I), nonspecifically bound proteins were eluted with buffer A (peak II), and bound proteins were eluted with buffer B (peak III).

The culture medium of insect Sf-21 cells infected with AcMNPV-gpl exhibited the ability to agglutinate washed rabbit red cells beginning ≈48 h postinfection (p.i.). This activity peaked ≈72 h p.i. (data not shown). Very little agglutinization activity was found in the supernatant of Sf-21 cells infected with AcMNPV-lacZ compared with that observed with supernatant from AcMNPV-gpl-infected Sf-21 cells. The expression of Gpl by AcMNPV-gpl was confirmed by Western blot analysis (Fig. 3, lane 5) by using polyclonal antibodies that were generated against Gpl expressed in bacteria. These antibodies also were used to prepare an immunoaffinity column that was used to purify the baculovirus-expressed Gpl (Fig. 2). Analysis of the immunoaffintiy-purified protein by SDS-PAGE gave a single band, with an approximate molecular mass of 33 kDa (Fig. 3).

Purification of Authentic Proteolytic Lectin from *G. austeni* Midguts and Bacterially Expressed Gpl. A two-step purification scheme was used to purify the authentic Gpl from midgut homogenates of *G. austeni* and recombinant Gpl expressed in bacteria. In the first step, the crude protein sample was applied onto a packed DEAE Sephacel column, and the bound proteins were eluted with a NaCl gradient (Fig. 4A). In the second step, Gpl that was eluted from the DEAE Sephacel column was applied onto a glucosamine affinity column and eluted with 0.2 M D-glucosamine in equilibration buffer (Fig. 4B). The sample purity was ascertained by SDS-PAGE (containing 2% β-mercaptoethanol), which yielded a single protein band (Fig. 5). The estimated molecular mass of the bacterially expressed Gpl was ≈32 kDa by SDS-PAGE.

Comparison of the Biological Activities of Authentic and Recombinant Gpl. The ability of the authentic (from the tsetse fly midguts) and recombinant (bacterially or baculovirus expressed) Gpls to agglutinate trypanosomes (purified from either in vivo or in vitro sources) and red blood cells are shown in Table 1. The

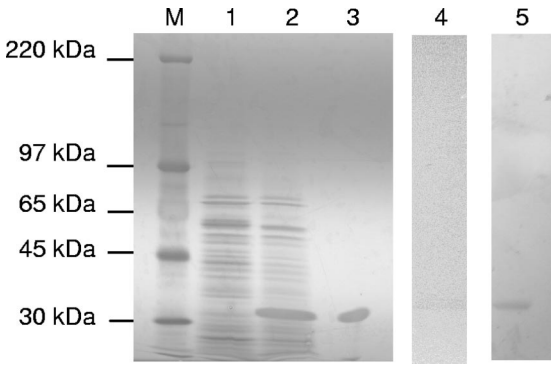

Fig. 3. SDS-PAGE (4–15%) analysis of proteins in the cell culture medium of Sf-21 cells infected with AcMNPV-LacZ (lane 1) or AcMNPV-gpl (lane 2) and affinity-purified, AcMNPV-gpl-expressed Gpl (lanes 3 and 4). Each lane was loaded with 15 μg of protein. The sizes of the molecular weight standards (lane M) are shown to the left. The proteins were visualized by silver staining (lanes M, 1, 2, and 3) or carbohydrate staining with PAS (lane 4). AcMNPV-gpl-expressed Gpl was separated by SDS-PAGE and then transferred onto nitrocellulose paper. The blot was then reacted with antiserum to the purified bacterial expressed Gpl (lane 5).

agglutination assay was carried out at 27°C because a previous study (Ibrahim et al. 1984) indicated that this was the optimum temperature for this assay. The agglutination titer reported is the reciprocal of the highest protein dilution that showed clumping of cells. The agglutination activities of the recombinant Gpls seemed to be identical, but only half as strong as that of the authentic Gpl. There was no difference in the agglutination of trypanosomes maintained by serial passage in rats (i.e., in vivo) or purified from blood (i.e., in vitro). The trypsinization activities of the authentic and recombinant Gpls are also shown in Table 1. The authentic Gpl showed higher (≈1.5-fold) trypsinization activity in comparison with that of the recombinant Gpls. The addition of 200 mM D-glucosamine to the assays strongly (60- to 250-fold) inhibited agglutinization activity. There was no significant difference between any of the three Gpl proteins in terms of the biological activities investigated

Partial Chemical Characterization of the Purified Baculovirus-Expressed Protein. The presence of covalently bound carbohydrate moieties on the purified baculovirus-expressed Gpl was determined by PAS staining after SDS-PAGE (Fig. 3, lane 4). The baculovirus-expressed Gpl was shown to be glycosylated on the basis of PAS staining.

Discussion

This report deals with the expression of a functionally active *Glossina* proteolytic lectin (Gpl) in insect cells by using a recombinant baculovirus expression vector. The gene encoding Gpl was obtained from a midgut-derived cDNA library from *G. austeni* and used to generate the recombinant baculovirus AcMNPV-gpl. The recombinant protein expressed by this virus was secreted into the culture medium of Sf-21 cells, indicating that the *G. austeni*-derived signal peptide was properly recognized in the lepidopteran-derived cells. Comparative sequence analysis, both at nucleotide and deduced amino acid levels, showed that the gene cloned from *G. austeni* is

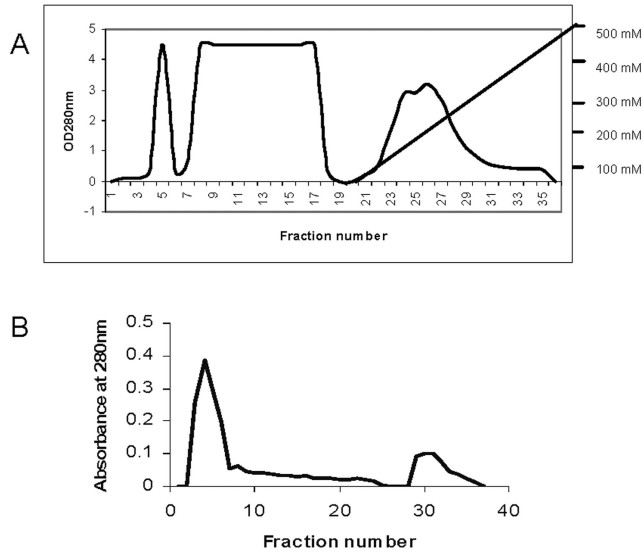

Fig. 4. Purification of bacterially expressed Gpl. (A) The lysate of *E. coli* cells transformed with pTriplEx.gpl was applied onto a DEAE Sephacel column. Unbound proteins (fractions 1–19) were removed from column with 20 mM Tris-Cl, pH 8.0, whereas bound proteins (fractions 21–36) were eluted using a salt gradient (0–0.5 M) in the same buffer. (B) Proteins eluted by the salt gradient were applied onto a BIS-equilibrated glucosamine affinity column. The column was washed with BIS to remove unbound material (fractions 1–26) and bound proteins (fractions 28–35) were eluted with 0.2 M glucosamine in BIS.

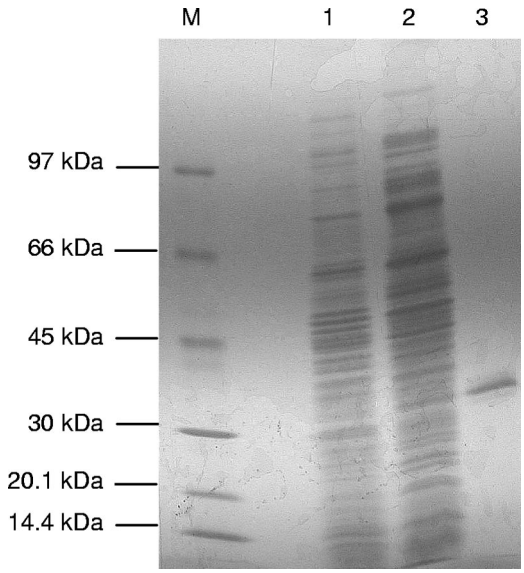

Fig. 5. SDS-PAGE (4–15%) analysis of the noninduced bacterial cell lysate (lane 1), pTriplEx2.gpl-transformed bacterial cell lysate (lane 2), and purified rGpl from pTriplEx2.gpl-transfromed bacterial cell lysate. Each lane was loaded with 30 μg of protein. The sizes of the molecular weight standards (lane M) are shown to the left. The proteins were visualized by Coomassie brilliant blue staining.

very similar to that previously reported for *G. fuscipes fuscipes*. This ties with the results of the purification of a proteolytic lectin from the midgut homogenate of both species.

The recombinant Gpls, expressed in insect Sf-21 cells and by bacteria, agglutinized washed trypanosomes and rabbit red blood cells. The recombinant Gpls produced an agglutination titer of 1,024, which was comparable with that obtained with the authentic Gpl from midgut homogenates. The recombinant Gpls also showed trypsinization activities (32.1–33.4 nmol/ml/min) against the substrate chromozym-TRY. This trypsinization activity was similar to that produced by the authentic Gpl (48.0 nmol/ml/min). Both agglutinization and trypsinization activities were strongly inhibited by D-glucosamine, a strong indication that the protein is a lectin. Recently, a bacterially expressed Gpl was characterized and shown to promote the transformation of *T. brucei brucei* in vitro (Abubakar et al. 2005). This transformation was observed with the aid of immunofluorescence microscopy.

In view of the drawbacks of vector-control measures that are currently in use, it has been suggested that the creation of transgenic tsetse flies that are refractory to trypanosomes will significantly contribute toward the control of trypanosomiasis, if used together with a sterile insect release program. At present, transgenesis of tsetse is not possible because its eggs are nurtured within the female and only released at the final stage of larval development. However, in a technique referred to as paratransgenesis (Crampton 1994, Aksoy et al. 2001), endosymbionts of the tsetse fly can be used to express a foreign gene within the adult fly. Thus, our findings may have use in terms of generating a transgenic endosymbiont that expresses Gpl in a paratransgenic insect. The expression of Gpl in a paratransgenic tsetse should produce the same effects as a transgenic Gpl-expressing insect.

PAS staining confirmed that the purified baculovirus-expressed Gpl was glycosylated. Interestingly, although there is a generally accepted view that *O*-linked and *N*-linked glycosylation occurs through serine and asparagines residues, respectively, this seems not to be the case in Gpl. The deduced amino acid sequence of gpl does not have the classical consensus signature sequences for these types of glycosylation. Recently, a number of studies (Haltiwanger et al. 1992, Previato et al. 1994, Hart et al. 1995, Mehta et al. 1997, Haynes 1998) have presented evidence for the existence of saccharide-protein linkages that do not follow the prevailing dogma. For example, in *Dictyostelium* spp. (Mehta et al. 1997), phosphoglycosylation occurs via the linkage of the sugar to a phosphate attached to a serine moiety on the protein backbone. However, there is no evidence as yet of a consensus recognition motif for this type of glycosylation (Haynes 1998). We intend to further characterize the baculovirus-expressed recombinant in terms of the structure of the sugar and the type of saccharide-protein linkage.

The lack of a carbohydrate moiety on the bacterially expressed Gpl had essentially no effect on its agglutinization and trypsinization properties. Although not

Table 1. Agglutination and trypsinization activities of the native and recombinant Gpls

Source of purified GPL	Agglutination activity[a]			Trypsinization activity[b] ± SD (nmol/ml/min)
	Parasites		Red blood cells	
	In vivo cultured	In vitro cultured		
Authentic	2,048	2,048	1,024	48.0 ± 3.0
Authentic + D-glucosamine	8	8	8	0
Bacterially expressed	1,024	1,024	512	32.1 ± 4.0
Bacterially expressed + D-glucosamine	8	8	8	0
Baculovirus expressed	1,024	1,024	512	33.4 ± 2.0
Baculovirus expressed + D-glucosamine	8	8	8	0

[a] Average value of two independent assays.
[b] Average value of three independent assays.

essential in these functions, glycosylation of Gpl may serve a structural role or ensure proper synthesis and positioning of Gpl within the organism. For example, appropriate glycosylation may be required for the proper targeting of the protein in the midgut region. The carbohydrate moiety of glycoproteins generally contributes a net negative charge and increased solubility to a protein (Nachon et al. 2002). Thus, it is also plausible that glycosylation plays a role in the proper solubility of Gpl within the midgut cells. Currently, Gpl is associated with two roles in the tsetse-trypanosome relationship (i.e., anti-trypanosome by causing an apoptosis-like cell death and pro-trypanosome by promoting establishment). This protein may have evolved as an adaptation by tsetse to reduce the physiological stress that is brought about by trypanosomes (Leak 1999). This also may explain why tsetse flies show low infectivity to trypanosome parasites in nature.

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