Identification, Cloning, and Characterization of Two N-Acetylgalactosamine-binding Lectins from the Albumen Gland of Helix pomatia

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Helix pomatia agglutinin (HPA), the lectin from the albumen gland of the Roman snail, has been used in histochemical studies relating glycosylation changes to the metastatic potential of solid tumors. To facilitate the use of HPA in a clinical (diagnostic) setting, detailed analysis of the lectin, including cloning and recombinant production of HPA, is required. A combination of isoelectric focusing, amino acid sequence analysis, and cloning revealed two polypeptides in native HPA preparations (HPAI and HPAII), both consistent with GalNAc-binding lectins of the H-type family. Pairwise sequence alignment showed that HPAI and HPAII share 54% sequence identity whereas molecular modeling using SWISS-MODEL suggests they are likely to adopt similar tertiary structure. The inherent heterogeneity of native HPA highlighted the need for production of functional recombinant protein; this was addressed by preparing His-thioredoxin-tagged fusion products in Escherichia coli Rosettagami B (DE3) cells. The recombinant lectins agglutinated human blood group A erythrocytes whereas their oligosaccharide specificity, evaluated using glycan microarrays, showed that they predominantly bind glycans with terminal α-GalNAc residues. Surface plasmon resonance with immobilized GalNAc-BSA confirmed that recombinant HPAI and HPAII bind strongly with this ligand (K_d = 0.60 nM and 2.00 nM, respectively) with a somewhat higher affinity to native HPA (K_d = 7.67 nM). Recombinant HPAII also bound the breast cancer cells of breast cancer tissue specimens in a manner similar to native lectin. The recombinant HPA described here shows important potential for future studies of cancer cell glycosylation and as a reagent for cancer prognostication.

Changes in the glycosylation of proteins are a feature of tumorigenesis and correlate with aggressive cancer cell behavior (1, 2). Aberrant, cancer-associated, glycosylation can be detected using carbohydrate-binding proteins, with the lectin from the Roman snail, Helix pomatia (HPA) having been shown to be particularly useful in this regard (3). Studies with HPA have shown a correlation between poor prognosis breast cancer that is metastatic to the axillary lymph nodes and lectin binding (4, 5). Further work has demonstrated that HPA binding is associated with aggressive colorectal (6, 7) and cervical (8) and gastric carcinoma (9). These observations led to the hypothesis that changes in glycosylation detected by HPA may be the same across a range of solid tumors (3). The utility of HPA for prognostication of solid tumors appears to lie in its ability to recognize the cancer-associated Tn-antigen (GalNAcα-O-Ser/Thr) and other, as yet undefined, epitopes (9). Tn-antigen expression occurs in a diverse range of neoplastic lesions and may, in part, be due to a loss of function of the Cosmc gene encoding a molecular chaperone required for T-synthase formation (10). This is associated with a reduction in synthesis of the core 1 disaccharide of O-glycans which are formed by the addition of Gal to the Tn-antigen to generate the T-antigen: Galβ1-3GalNAcα1-Ser/Thr (11). Unfortunately, despite the apparent utility of HPA for cancer prognostication, the lectin has yet to find a place in clinical decision making.

An initial attempt to solve the three-dimensional structure of HPA was reported in 2000 (12), but the limited resolution (3.6 Å) and lack of amino acid sequence prevented the structure from being solved (12). In 2006, HPA crystals diffracting to 1.3 Å allowed the sequence and structure to be determined (13). This confirmed earlier findings (14–17) that described HPA as a hexamer formed by disulfide bonds linking dimers, which then form a trimer of dimers by noncovalent interactions. The crystal structure showed the carbohydrate binding site is situated at the interface between two monomers. The novelty of the structure of the binding pocket led to the assignment of a new lectin family, the H-type, named after H. pomatia. At this time, another polypeptide sequence with a 54% sequence identity to the lectin was deposited in the EMBL DNA sequence data base (accession number: AJ639657 (Uniprot accession number: Q57553)) by Gerlach and Schmidt who described this polypeptide as H. pomatia agglutinin. However, because it only showed 54% sequence identity to HPA, Sanchez et al. (13) suggested that this new protein may have derived from a different snail species. A further possibility was that this protein represented a second lectin in the HPA preparation.

Our initial analysis of HPA suggested that native HPA was more complex than a single polypeptide, so we set out to identify the composition of the material. In addition, we recognized that further analysis of the sequence and structure of HPA would provide important insights into the carbohydrate-binding properties of the lectin and that this information could be...
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used to prepare a recombinant form of the polypeptide, the availability of which might lead to the development of new tools for cancer diagnosis and, potentially, therapy.

EXPERIMENTAL PROCEDURES

Preparation of HPA—H. pomatia snails were obtained in May 2005 with the kind assistance of Dr. Anthony Leathem, Royal Free and University College Medical School, London, from a wood in Oxfordshire, England. Snails were maintained in the Cancer Glycobiology Laboratory at the University of Westminster in a screened terrarium, in artificial laboratory daylight, at 20 °C and were fed lettuce and water ad libitum. The snails were anesthetized and deshelled, and the albumen gland was dissected. HPA was purified from the albumen gland using the method of Vretblad et al. (18). In brief, albumen glands from 10 snails were minced in 10 ml of 10 mM sodium phosphate buffer, pH 7.2, containing 150 mM NaCl (PBS) per gram of albumen gland, and the lectin HPA was purified on a 1-ml N-acetylglactosamine (GalNAc)-agarose affinity chromatography column eluted with 68 mM N-acetylgalactosamine (GlcNAc).

SDS-PAGE—SDS-PAGE was performed using a Tris-Tri-cine-SDS electrophoresis buffer system (19) with step gradients of 15, 10, and 5% polyacrylamide; or with the Tris-glycine-SDS system for analysis of rHPAI-Trx/rHPAII-Trx which was performed according to Ref. 20. Samples were boiled for 5 min or treated with 50 mM dithiothreitol (DTT) where appropriate. After electrophoresis, the proteins were visualized by staining with 0.025% (w/v) Coomassie Brilliant Blue R-250.

Two-dimensional Electrophoresis—To separate the individual subunits of HPA a combination of isoelectric focusing and SDS-PAGE was used (21). 1 mg of HPA lectin was solubilized in 100 µl of a solution containing 8 mM urea and 400 mM NH₄HCO₃, pH 8.1. An equal volume of 50 mM DTT was added and the mixture incubated for 15 min at 20 °C followed by addition of 9 µl of 100 mM iodoacetamide with a further incubation for 15 min. The protein solution was separated on a 7-cm linear immobilized pH gradient strip at 300 V for 30 min, 600 V for 45 min, and 3500 V for 2.45 h, at 20 °C. After the isoelectric focusing step the strip was embedded on the top of an SDS-polyacrylamide gel using molten agarose (1% w/v in Tris-Tricine-SDS cathode buffer) and subjected to electrophoresis as described previously.

MS/MS—Mass spectrometry of the protein spots from the 2-DE was performed using an Applied Biosystems 4700 Proteomics Analyzer (MALDI-TOF-TOF) by commercial arrangement with Dr. J. Thomas, School of Biology, University of York, UK. Protein spots were excised and digested in gel with trypsin, and a mixture of the resulting peptides and α-cyano-4-hydroxycinnamic acid (3.6 mg/ml) in 10 mM ammonium citrate was spotted onto a MALDI plate and allowed to air dry. MS measurements were collected using a 4700 Proteomics Analyzer Discovery System (Applied Biosystems).

mRNA Extraction and Cloning of HPA Genes—RNA was isolated from the freshly excised albumen gland of H. pomatia using a combination of TRizol Reagent (22) and PolyATtract mRNA Isolation System (Promega); reverse transcription of RNA to cDNA was performed using an Omniscript reverse transcriptase kit (QIagen). RT-PCR using the cDNA prepared above and degenerate oligonucleotide primers designed following analysis of the protein sequences determined by MS/MS (see above) was used to amplify the HPA gene(s), and the ampli-cions were then ligated into the pGEM-T vector (Promega), transformed into E. coli DH5α T1 cells, and recombinants were selected using isopropyl 1-thio-β-d-galactopyranoside (100 mM) and 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (20 µg/ml) selection. Putative positive colonies were evaluated using PCR and sequenced at the Advanced Biotechnology Centre, Imperial College, London. To obtain the full-length genes for the HPA proteins the positive clones were extended using dGTP and terminal transferase, and the product was amplified by PCR using the Phusion system (New England Bios-labs) with ~10 ng of template DNA, cloned into pGEM-T, and resequenced.

Production of Recombinant HPAI and HPAII Proteins—Recombinant HPAI and HPAII proteins were prepared as both histidine-tagged (rHPAI-His/rHPAII-His) and thioredoxin protein-tagged (rHPAI-Trx/rHPAII-Trx) fusion proteins using the pET-32a vector system (supplemental data 1), expressed in E. coli Rosetta-gami B (DE3) pLysS-competent cells (Novagen), supplemented with 100 µg/ml ampicillin, 34 µg/ml chloramphenicol, 15 µg/ml kanamycin, and 12.5 µg/ml tetracycline. Fresh grown colonies containing the recombinant plasmids were used to inoculate 50 ml of LB medium containing the appropriate antibiotics and grown overnight at 37 °C with shaking at 250 rpm, and 500 ml of prewarmed LB medium, containing the four antibiotics as before, was then inoculated with 25 ml of the overnight culture and grown at 37 °C until the A₆₀₀ nm reached 0.5 after which the culture was cooled on ice for 30 min. Isopropyl 1-thio-β-d-galactopyranoside was then added to a final concentration of 1 mM, and the culture was grown at 18 °C for 18 h with shaking at 250 rpm. Cells were harvested by centrifugation and resuspended in 50 ml of 20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10 mM imidazole, 0.1% v/v Triton X-100, 10 µg/ml lysozyme, and 0.1 mM phenylmagnesiumsulfonate fluoride (PMSF). The cell suspension was incubated on ice for 30 min, and the cells were lysed by sonication on ice (6 × 30 s at 20 kHz at 40% power) using a MS73 Status 200.

Recombinant proteins were purified in two steps, using nick-el-agarose (GE Healthcare) and GalNAc-agarose columns (Sig-ma-Aldrich). Chromatography was carried out at 20 °C with buffer flow rates set to 1 ml/min. For nickel affinity chromatography, the E. coli cell lysate was loaded onto the column in 20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 20 mM imidazole, washed with 10 column volumes of the same buffer, and the recombinant protein was eluted with 5 column volumes of the same buffer containing 500 mM imidazole. For the second purification step, a GalNAc-agarose affinity column was loaded with the fractions containing the recombinant protein from the nickel chromatography step, unbound material was washed from the column with 5 column volumes of PBS, and recombinant protein was eluted from the column using 5 column volumes of PBS containing 250 mM GlcNAc. The purified proteins were dialyzed overnight against PBS at 4 °C, concentrated by centrifugation using the Centricon system using 5-kDa molec-
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ular mass cut-off (Millipore) and evaluated using SDS-PAGE (see above).

**Agglutination Assays**—Human erythrocytes with blood groups A, B, and O (National Blood Service, London, UK) were used as a 10% (v/v) suspension in PBS after washing three times by centrifuging 5 ml of blood at 1,500 × g for 5 min at 4 °C. Agglutination assays were conducted in 96-well round bottom plates (preblocked overnight at 4 °C with 1% (w/v) BSA in PBS). 5-μl aliquots of native HPA, rHPAI-Trx, or rHPAII-Trx solutions were mixed with 5 μl of PBS and 10 μl of 10% erythrocyte suspension in each test well. The 96-well plate was rocked at 20 °C for 1 h, and the extent of the agglutination was assessed using an inverted microscope and scored from (−) no agglutina-
tion to (+++) complete agglutination.

**Analysis of Lectin Binding Using Surface Plasmon Resonance (SPR)**—SPR analysis was carried out using a BIAcore 2000 using a CM5 biosensor chip (GE Healthcare). Ligands were immobilized onto the surface of the chip using an amine coupling reaction, as described in the instructions provided by the manufacturer (GE Healthcare), creating the following linkages: (i) reference cell (no ligand coupled); (ii) BSA; (iii) GalNAc linked to BSA via a 14-carbon linker spacer (Dextra Laboratories), (iv) rHPAI-Trx (GE Healthcare), was used for analysis of the SPR data. Analysis of HPA lectin purified from the albumen gland of the Roman snail, *H. pomatia*, highlighted the complexity of the preparation. A, separation using Tris-Tricine SDS-PAGE at 180 V for 35 min. The lanes indicate native HPA, HPA after denaturation for 5 min at 100 °C, and HPA after treatment with 0.2 M DTT for 15 min at 20 °C. 25 μg of protein was loaded into each well. The protein was visualized by staining the gel with Coomassie Brilliant Blue. B, two-dimensional separation of 150 μg of HPA preparation using 3–10 pH linear immobilized pH gradient strip and SDS-PAGE as for A. Spots indicated by the arrows and identified by Coomassie Brilliant Blue staining were used for MS/MS analysis. C, amino acid sequence data for spots 3, 4, and 14 as indicated.

**RESULTS**

**HPA Heterogeneity**—We set out to analyze HPA because earlier studies hinted that the material may comprise a mixture of proteins (18, 25). HPA was purified from freshly dissected snail albumen glands using GalNAc-agarose resin, and the resultant preparation was analyzed using a Tris-Tricine SDS-PAGE system (Fig. 1A). In accordance with previous reports, a polypeptide of 79 kDa was observed (15, 18, 26). When the lectin was heated at 100 °C for 5 min the appearance of a band of 26 kDa consistent with the molecular mass of two monomers (each 13 kDa) was detected. Treatment of the preparation with DTT was expected to generate a single band consistent with the monomer on SDS-PAGE analysis. Somewhat surprisingly, two bands of ~13 kDa as well as a band of 26 kDa were apparent. Initially, we thought that the two bands might represent glycosylated variants of the same peptide. Glycopeptides, however, tend to resolve poorly on SDS-PAGE due to the minor differences in the molecular mass of the species present. As the peptides we observed in the native HPA preparation separated into two sharp bands on SDS-PAGE, this was the first piece of evidence suggesting that the peptides probably derived from different amino acid sequences, as a dimer of identical monomers should not yield two similar but rather two identical species.

When the HPA preparation was analyzed by two-dimensional electrophoresis, >20 polypeptide species were detected, ranging from 8 to 13 kDa (Fig. 1B). A similar observation was made using HPA purchased from Sigma with some differences in the relative spot intensities (data not shown). Peptide mass fingerprint analysis was performed with peptides derived from the isoelectric focusing-separated polypeptides, but no significant hits from the GenBank/EBI data bank search were obtained. When MS/MS analysis was used, four peptide sequences were obtained from the analysis of species 3 and 4.
Cloning of HPAI and HPAII Genes—The peptide GEIDCGSDSSWP elucidated from sequencing species 14 was used to design degenerate oligonucleotide primers to allow the cloning of the HPA gene which was named HPAI. The sequence of HPAI was found to be identical to a sequence in the EMBL DNA sequence data base (accession number CAG26659) deposited by Gerlach and Schmidt. It become apparent that peptides from species 3 and 4 (Fig. 1B) did not originate from this sequence and that these might represent a new protein. To investigate this possibility, degenerate oligonucleotide primers corresponding to the CGNDAG sequence were prepared and used to clone a gene for a second HPA protein known as HPAII. The sequence of HPAII is identical to that deposited in GenBank by Sanchez et al. (accession number DQ341310) (13).

The predicted molecular masses of HPAI and HPAII are 10,861 and 10,662 Da, respectively, and these corresponded broadly to the bands observed during SDS-PAGE analysis of the native protein (Fig. 1A). The slight differences in molecular mass are potentially attributable to post-translational modifications such as glycosylation. The isoelectric points for HPAI and HPAII are predicted to be 6.77 and 8.47, respectively; again, these were within the range of the isoelectric points observed for the HPA polypeptides that were obtained during the two-dimensional electrophoresis analysis (Fig. 1B).

Pairwise sequence alignment of HPAI and HPAII showed that the two sequences share 54% sequence identity (Fig. 2A). Homology models of HPAI (based on PDB 2CE6, native HPAII and 2CCV, HPAII with GalNAc soaked into the crystal) were generated using SWISS-MODEL and allowed the structural similarity between the two proteins to be assessed in silico (Fig. 2B).
The only apparent difference between the carbohydrate binding site of HPAI and HPAII is at position 24 with asparagine in HPAI and glycine in HPAII. This substitution would not be expected to interfere with the carbohydrate binding properties of the lectin because only the nitrogen of the main chain of the amino acid participates in hydrogen bonding with the O of the acetyl group of the carbohydrate. Analysis of the amino acid sequence for HPA-I indicated that, in contrast to HPA-II, it does not possess an N-linked glycosylation consensus sequence at Asn^55.

Functional Analysis of HPAI and HPAII—HPAI and HPAII were successfully prepared as His and Trx fusion proteins (Fig. 3A). To check whether the addition of the Trx tag affected the structure of the protein, the migration of rHPAI-Trx/rHPAII-Trx was evaluated by separation on PAGE (Fig. 3B). In this analysis, both rHPAI-Trx and rHPAII-Trx were observed as trimeric proteins, held together with intermonomer disulfide bridges, that could be broken by heating to boiling for 5 min or by the addition of DTT.

The rHPAI-Trx and rHPAII-Trx fusion proteins agglutinated human erythrocytes of blood group A in a similar manner to native HPA (Fig. 3C). Concentrations of lectin <2.5 μg/ml were insufficient to result in agglutination, even after several hours of incubation, whereas at ≥12.5 μg/ml, agglutination was
observed within seconds. GalNAc and GlcNAc competitively inhibited both lectin agglutination reactions when used at 25 mM (Fig. 3C). rHPAI-Trx and rHPAII-Trx did not agglutinate erythrocytes of blood group B or O (data not shown).

The oligosaccharide binding properties of the recombinant lectins were analyzed further on the printed glycan microarray of the Consortium for Functional Glycomics, which comprised 320 different immobilized glycan structures (Fig. 4 and supplemental data 3). In this system rHPAI-Trx and rHPAII-Trx recognized almost identical glycan residues and showed a preference for glycans containing terminal α-linked GalNAc; with GalNAcα1→3Gal > GalNAcα1→4Gal. rHPAI-Trx and rHPAII-Trx did not bind oligosaccharides containing sialic acid, but in all other respects the native and recombinant material showed the same glycan binding on the array. The native HPA preparation recognized the same oligosaccharides (although with different intensities) as have previously been reported by Sanchez et al. (13).

The binding of rHPAI-His to breast cancer tissue was evaluated using a breast cancer tissue array and compared with native HPA and showed that rHPAI-His binds to cancer tissue samples in a manner similar to that observed with the native lectin (Fig. 6).

**DISCUSSION AND CONCLUSIONS**

In this study the lectin HPA was found to be a heterogeneous mixture of polypeptides coded for by separate genes expressed in the albumen gland of *H. pomatia*. It has been proposed that the main physiological function of HPA in the snail is to protect the eggs by binding and agglutinating pathogens on their surface (26, 27). HPA has also been shown to enhance phagocytosis of foreign cells by *H. pomatia* hemocytes (25). Consistent with these findings are the diverse range of glycan structures that HPA bound on the glycan microarray. In addition, the analysis showed that although native HPA recognizes many glycosylated epitopes (including α-GalNAc/α-GlcNAc and sialic acid), the recombinant material only bound glycans terminating in α-GalNAc/α-GlcNAc. It is possible that native HPA binds to NeuNAc-containing oligosaccharides on the CFG array by virtue of a contaminant in the native lectin preparation. The structural analysis by Sanchez et al. (13) discounted the possibility of a secondary binding site on the lectin. A further possibility is the presence of another polypeptide in the HPA preparation capable of binding sialylated residues (28).
In silico structural analysis, through a comparison of the x-ray crystallography data of HPAII (13) and molecular modeling, have enabled the putative three-dimensional structure of HPAI to be determined. These investigations show that HPAI belongs to the H-type lectin family and that GalNAc will fit in the binding pocket with the same orientation in both polypeptides, HPAI and HPAII.

Recombinant forms of both of the polypeptides have been produced, and analysis confirmed HPAI and HPAII as GalNAc-binding lectins. Both HPAI and HPAII bind to GalNAc with approximately equal affinity and in a similar order of magnitude as that reported by Oyelaran et al. (29) who studied the interaction between HPA with GalNAc (at various coating densities). The BioCave data were also consistent with the results of the “end-point” agglutination assay where both proteins performed in a similar manner. Clearly, further work is required to fully characterize the proteins more extensively, but the data obtained thus far suggest similar binding characteristics for the two polypeptides.

Our initial analysis using a breast cancer tissue array showed that rHPAI-His binds breast cancer cells. The focus of future work will be identification of the cancer-associated glycans and glycoproteins recognized by HPA. Studies focused on the binding of recombinant HPA to cancer tissues will be an important next step in driving forward method development efforts directed toward the practical use of recombinant HPAI and HPAII for cancer diagnosis and prognostication.

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