Glycoconj J (2007) 24:143–156
DOI 10.1007/s10719-006-9022-z

The *Sclerotinia sclerotiorum* agglutinin represents a novel family of fungal lectins remotely related to the *Clostridium botulinum* non-toxin haemagglutinin HA33/A

Els J. M. Van Damme · Sachiko Nakamura-Tsuruta · Jun Hirabayashi · Pierre Rougé · Willy J. Peumans

Received: 26 June 2006 / Revised: 11 November 2006 / Accepted: 14 November 2006 / Published online: 9 February 2007

Abstract Previous studies indicated that sclerotes of the phytopathogenic Ascomycete *Sclerotinia sclerotiorum* contain a lectin that based on its molecular structure, specificity and N-terminal amino acid sequence could not be classified yet into any lectin family. Using a combination of molecular cloning, frontal affinity chromatography and molecular modelling the identity of the *S. sclerotiorum* agglutinin (SSA) was analyzed. Molecular cloning demonstrated that SSA shares no sequence similarity with any known fungal lectin or protein. The lectin is synthesized as a 153 amino acid polypeptide without signal peptide and undergoes apart from the removal of the N-terminal methionine no further processing. Frontal affinity chromatography revealed that the binding site of SSA primarily accommodates a non-reducing terminal GalNAc with a preference for the α- over the β-anomer. SSA also strongly interacts with both glycolipid type glycans with terminal non-reducing Gal or GalNAc and galactosylated N-glycans. SSA shares a residual sequence similarity with part of the non-toxin haemagglutinin HA33/A from *Clostridium botulinum*. Molecular modeling using the three-dimensional structure of HA33/A as a template indicated that SSA can fold into a similar β-trefoil domain. Though these results should be interpreted with care it is tempting to speculate that the Sclerotiniaceae lectins thus appear to be structurally related to the ricin-B superfamily. All evidence suggests that SSA represents a novel family of fungal lectins with a unique sequence and sugar-binding properties. Taking into account that orthologues of SSA are fairly common within the family Sclerotiniaceae but could not be identified in any other fungal species one can reasonably conclude that SSA-type lectins are confined to a small taxonomic group of the Ascomycota.

Keywords *Sclerotinia sclerotiorum* agglutinin · Fungal lectin · N-glycan · Novel protein family · Molecular cloning · Sugar specificity

Abbreviations

AOL	*Arthrobotrys oligospora* lectin
BFA	*Botryotinia fuckeliana* agglutinin
CCL	*Ciborinia camelliae* lectin
FAC	frontal affinity chromatography
HCA	Hydrophobic Cluster Analysis
ORF	open reading frame
PA	pyridylaminated
PNP	*p*-nitrophenyl
SSA	*Sclerotinia sclerotiorum* agglutinin

E. J. M. Van Damme (✉) · W. J. Peumans
Department of Molecular Biotechnology,
Lab. Biochemistry and Glycobiology,
Ghent University, Coupure Links 653,
9000 Ghent, Belgium
e-mail: ElsJM.VanDamme@UGent.be

S. Nakamura-Tsuruta · J. Hirabayashi
Glycostructure Analysis Team Research Center for Glycoscience,
National Institute of Advanced Industrial Science and Technology,
AIST Tsukuba Central 2, 1-1-1, Umezono,
Tsukuba, Ibaraki 305-8568, Japan

P. Rougé
Surfaces cellulaires et Signalisation chez les Végétaux,
UMR CNRS-UPS 5546, Pôle de Biotechnologies Végétales, 24
Chemin de Borde Rouge, B.P. 17,
31326 Castanet-Tolosan, France

Introduction

Many mushrooms and other fungi contain carbohydrate-binding proteins commonly known as lectins. Though not

all previously identified lectins have been purified and characterized the available biochemical, sequence and structural data provide ample evidence that fungi express a heterogeneous mixture of carbohydrate-binding proteins. In contrast to animal and plant lectins [1, 2], the evolutionary relationships between the different fungal lectins are only partly established. Accordingly, no comprehensive system has been elaborated yet to classify the fungal lectins in families of structurally and evolutionary related proteins. For the same reason, it is difficult to assess for most fungal lectins their possible relationships to bacterial, animal and plant lectins.

Hitherto, different types of fungal lectins have been identified with certainty on the basis of complete sequence data. Classical examples are the *Agaricus bisporus* [3, 4] and *Aleuria auarantia* [5] type lectins and the fungal galectins [6, 7]. More recently, lectins from the mushrooms *Marasmius oreades* [8] and *Polyporus squamosus* [9] were recognized as members of the ricin-B family. Cloning and structural analysis revealed that the hemolytic *Laetiporus sulphureus* lectin also contains a distantly related ricin-B domain but due to the presence of an in tandem arrayed hemolytic domain should be classified into a distinct (sub) family [10, 11]. Other recently discovered families of fungal lectins are represented by the mycelial aggregate-specific lectins from *Pleurotus cornucopiae* [12], the *Grifola frondosa* fruit body lectin [13], the *Flammulina velutipes* immunomodulatory protein [14], the lectin from *Rhizopus stolonifer* [15], and the *Psathyrella velutina* fruit body lectin [16]. In addition, putative expressed proteins closely related to the snowdrop (*Galanthus nivalis*) lectin family have been identified in several fungal species [17, 18]. Moreover, partial sequence data indicate that several other fungal lectins like these found in *Paracoccidioides brasiliensis* [19], *Lyophyllum shimeiji* [20] and *Polyporus adusta* [21] can not be classified into any of these families, which implies that fungi express at least 11 different types of carbohydrate-binding proteins. Some of these fungal lectins are definitely related to bacterial, animal or plant lectins whereas others are apparently confined to fungi. This suggests that only part of the carbohydrate binding domains found in fungi were acquired through vertical inheritance from a prokaryotic or early eukaryotic ancestor and that the remaining part was most probably developed by fungi themselves.

To further corroborate the diversity of fungal lectins a previously isolated [22] but still unclassified lectin [23] from the phytopathogenic Ascomycete *Sclerotinia sclerotiorum* was cloned and further characterized. The *S. sclerotiorum* agglutinin (SSA) represents a novel family of fungal lectins that shares no detectable sequence similarity with any other fungal protein but is remotely related to the non-toxin haemagglutinin HA33/A from *Clostridium botulinum*. Closely related lectins occur in several other species of the family Sclerotiniaceae but are apparently absent from all other completed or nearly completed fungal genomes. The relevance of these findings for the elucidation of the molecular evolution of fungal lectins is discussed.

Materials and methods

Material and growth conditions

S. sclerotiorum (strain S1954 kindly provided by Prof. M. Höfte, Ghent University, Belgium) was grown at room temperature (20–25°C) on potato dextrose agar medium.

Oligosaccharides

p-Nitrophenyl (pNP) glycosides of Galβ, GalNAcβ, Manα, GlcNAcα, Fucα and Galβ1-4Glcβ were purchased from Sigma (St Louis, MO, USA). Galα, GalNAcα, Manβ, Galβ1-4GlcNAcβ, Galβ1-3GalNAcα (Core1), GlcNAcβ1-6(Galβ1-3)GalNAcα (Core2), GlcNAcβ1-3GalNAcα (Core3) and GlcNAcβ1-6GalNAcα (Core6) were obtained from Toronto Research Chemicals, Inc. (North York, Canada), Glcα was from Calbiochem (San Diego, CA, USA), Forssman pentasaccharide was from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and Glcβ and GlcNAcβ from Seikagaku Co. (Tokyo, Japan). Pyridylaminated (PA) oligosaccharides used in this study are listed in Fig. 1. The suppliers have been described in previous report [24].

Purification of the *Sclerotinia sclerotiorum* agglutinin

The *S. sclerotiorum* agglutinin was isolated from mature sclerotes by affinity chromatography on galactose-Sepharose 4B according to previously described procedures [22].

Analytical techniques

Crude extracts were analyzed by SDS-PAGE in 15% acrylamide gels as described by Laemmli [25]. Proteins were visualized by staining with Coomassie brilliant blue R250.

Isolation of RNA from sclerotes

Sclerotes were frozen and ground in liquid nitrogen using mortar and pestle. RNA was extracted using the FastRNA Pro Green kit in an automatic homogenizer (FastPrep Instrument, MP Biomedicals and Qbiogene, Irvine, CA, USA) following the manufacturer's recommendations.

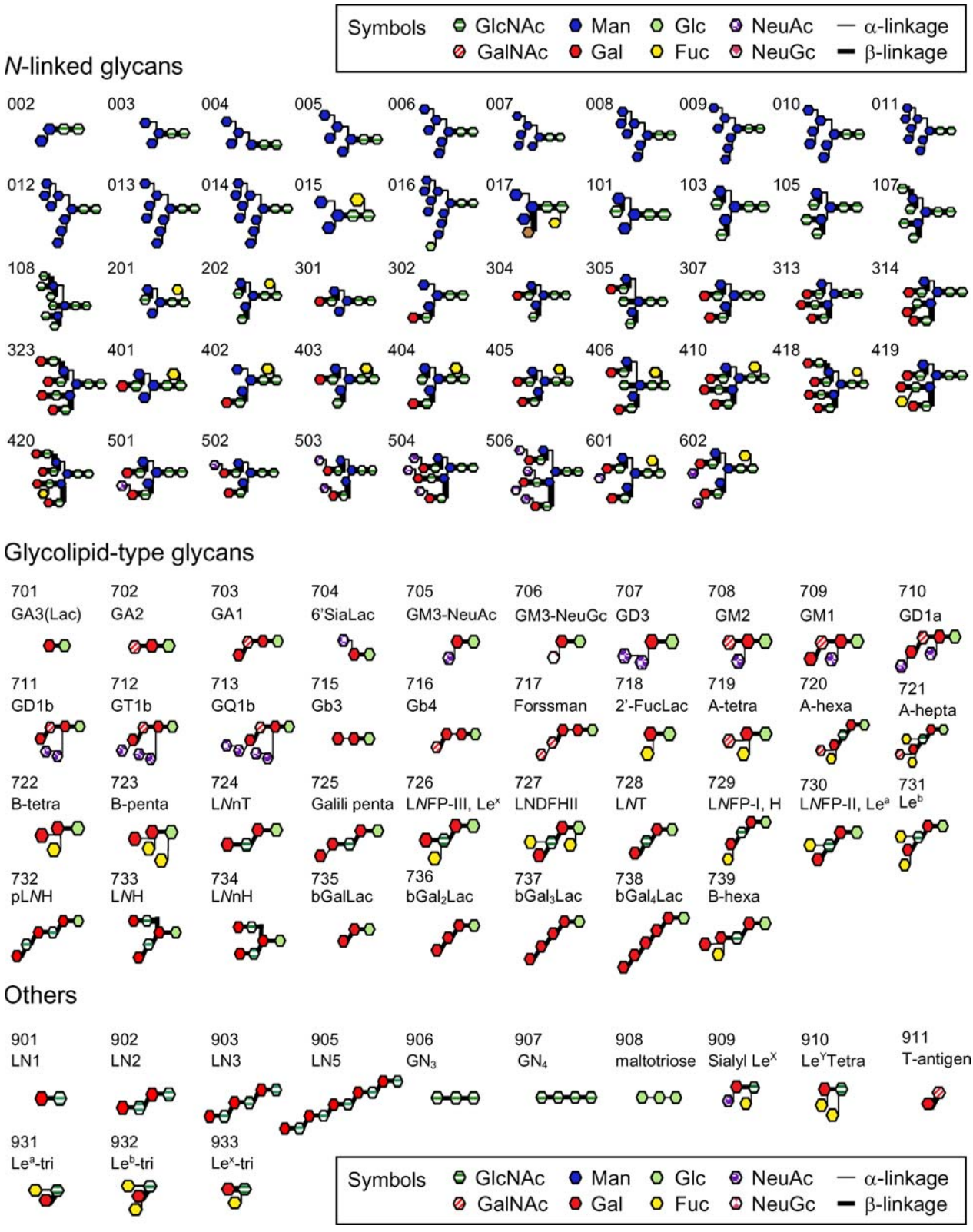

Fig. 1 Schematic representation of the structure of the glycans used for the determination of the sugar specificity of SSA. All pyridylaminated glycans tested in this study are shown. *Symbols* used to represent pyranose rings of monosaccharides and *bars* used to indicate linkage are explained in the box at the *bottom* of the figure. Anomeric carbon, *i.e.*, position 1, is placed at the *right hand side*, and 2, 3, 4... are placed *clockwise*. *Thin* and *thick bars* represent α and β carbons, respectively

RT-PCR and PCR amplification of cDNA sequence encoding SSA

cDNA was synthesized from the isolated RNA using the 1st Strand cDNA Synthesis kit for RT-PCR (AMV) (Roche Applied Science). cDNA sequences encoding SSA were amplified by PCR using primers derived from the N- (5′ATGGGCTTTAAGGGCGTTGG 3′) and C-terminal (5′ CTACTTGGCGTCAAAGTACC 3′) sequence of the coding sequence found in *S. sclerotiorum* 1980 cont1.457, whole genome shotgun sequence (Genbank Accession No. AAGT01000457). The reaction mixture for amplification of cDNA sequences contained 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 100 mg l^{-1} gelatin, 0.4 mM of each dNTP, 2.5 units of Taq polymerase (Invitrogen, Carlsbad, CA), 5 μl of cDNA and 20 μl of the appropriate primer mixtures (5 μM), in a 25 μl reaction volume. After denaturation of the DNA for 5 min at 95°C amplification was performed for 30 cycles through a regime of 15 s template denaturation at 92°C followed by 30 s primer annealing at 55°C and 1 min primer extension at 72°C. The PCR fragments were cloned in pCR2.1-TOPO cloning vector using the TOPO TA cloning kit from Invitrogen (Carlsbad, CA). Plasmids were isolated from purified single colonies on a miniprep scale using the alkaline lysis method [26] and sequenced by the dideoxy method [27]. The cDNA sequence of SSA has been deposited in Genbank under the Accession No. DQ468383.

Frontal affinity chromatography analysis

Purified SSA was dissolved in 50 mM $NaHCO_3$ buffer, pH 8.3, containing 0.5 M NaCl and coupled to NHS-activated Sepharose 4FF (Amersham Pharmacia Biotech, Bucks, UK) according to the manufacturer's instructions. After blocking of excess NHS groups by 1 M monoethanolamine followed by extensive washing, the SSA-Sepharose was suspended in Tris–buffered saline (TBS: 10 mM Tris–HCl pH 7.4. containing 0.15 M NaCl) and packed into a capsule-type miniature column (inner diameter, 2 mm; length, 10 mm; bed volume, 31.4 μl) (Shimadzu Co., Kyoto, Japan). The amount of immobilized lectin was 5 mg/ml (as estimated by quantifying the protein recovered in the wash fraction using a dye-binding assay) [28].

Frontal affinity chromatography (FAC) was performed using an automated FAC system (FAC-1, Shimadzu Co.) as described previously [24, 29]. Briefly, the column of SSA-Sepharose 4FF was mounted into a stainless holder and connected to the FAC-1 apparatus. The column was equilibrated with TBS at a flow rate of 0.125 ml/min at 25°C. After equilibration, either PA- (2.5 nM or 5.0 nM) or pNP-oligosaccharides (5.0 μM) dissolved in TBS were successively injected onto the SSA-column using an automated mode. Elution of PA-oligosaccharides was monitored by fluorescence (excitation and emission wavelengths of 310 and 380 nm, respectively), whereas pNP-glycosides were detected by UV absorption at 280 nm. The elution front (V) was calculated according to the method originally described by Arata et al. [30]. Retardation of the elution front ($V-V_0$) relative to that of an appropriate standard (i.e., $Man_3GlcNAc_2$-PA or Fucα-pNP, V_0) was calculated according to basic equation of FAC, Eq. 1, where B_t is the effective ligand content (expressed in mol) and $[A]_0$ is the initial concentration of PA-oligosaccharide [30–33]. Equation 1 can be simplified into Eq. 2, where $[A]_0$ (e.g., $< 10^{-8}$ M) is negligibly small as compared to K_d (e.g., $> 10^{-6}$ M).

$$K_d = B_t/(V - V_0) - [A]_0 \quad (1)$$

$$K_d = Bt/(V - V_0), \text{if } K_d >> [A]_0 \quad (2)$$

Retrieval of sequences

Sequences encoding SSA-like proteins were retrieved by BLAST searches using both the amino acid and nucleotide sequences of SSA as a query. The following databases were screened for the presence of EST and/or genomic sequences encoding fungal proteins sharing sequence similarity with SSA: National Center for Biotechnology Information (NCBI: http://www.ncbi.nlm.nih.gov/), Consortium for the Functional Genomics of Microbial Eukaryotes (COGEME: http://www.cogeme.man.ac.uk/): Phytopathogenic Fungi and Oomycete EST Database, The Institute for Genomic Research (TIGR: http://tigrblast.tigr.org/tgi/), Saccharomyces Genome database (SGD™: http://www.yeastgenome.org/), *Fusarium graminearum* Database (http://www.broad.mit.edu/annotation/fungi/fusarium) and *Aspergillus oryzae* EST DataBase (http://www.nrib.go.jp/ken/EST/db/).

Molecular modeling

Multiple amino acid sequence alignments were carried out with CLUSTAL-X [34] and displayed with ESPript [35] using the Risler's structural matrix for homologous amino acid residues [36].

Hydrophobic Cluster Analysis (HCA) [37] was performed to delineate the conserved secondary structural features (strands of β-sheet) along the amino acid sequences of SSA and BFA using the neurotoxin-associated protein HA33/A from *C. botulinum* (RSCB PDB code 1YBI) [38] as a model. HCA plots were generated using the HCA server (http://www.lmcp.jussieu.fr).

Molecular modeling of SSA and BFA was carried out on a Silicon Graphics O2 R10000 workstation, using the programs InsightII, Homology and Discover3 (Accelrys, San Diego CA, USA). The atomic coordinates of the *C. botulinum* HA33/A protein (RCSB PDB code1YBI) [38] were used to build the three-dimensional model of the fungal lectins. Although the identity and similarity between SSA/BFA and 1YBI is only 12 and 50%, respectively, HCA plots were sufficiently similar to build fairly accurate three-dimensional models using the X-ray coordinates of the *C. botulinum* protein as a template. Steric conflicts were corrected during the model building procedure using the rotamer library [39] and the search algorithm implemented in the Homology program [40] to maintain proper side-chain orientation. The geometry of loop regions was corrected using the refine option of TurboFrodo [41]. An energy minimization of the final models was carried out by 300 cycles of steepest descent using Discover3. The program TurboFrodo was run to draw the Ramachandran plot and to perform the superposition of the model with the template protein. PROCHECK [42] was used to assess the geometric quality of the three-dimensional models. All the residues of SSA and BFA were correctly assigned on the best allowed regions of the Ramachandran plot with the exception of residues Trp24 and His69 of SSA, and residues Leu53 and Val54 of BFA, which are located in the disallowed regions of the plot (result not shown). Cartoons were drawn with PyMOL (W. L. DeLano (http://www.pymol.org)).

Electrostatic potentials were calculated and displayed with GRASP using the parse3 parameters [43]. The solvent probe radius used for molecular surfaces was 1.4 Å and a standard 2.0 Å-Stern layer was used to exclude ions from the molecular surface [44]. The inner and outer dielectric constants applied to the protein and the solvent were respectively fixed at 4.0 and 80.0 and the calculations were performed keeping a salt concentration of 0.145 M.

Results

Molecular cloning of SSA

The *S. sclerotiorum* agglutinin was previously identified as one of the major soluble proteins in mature sclerotes and described as a homodimeric protein built up of subunits of approximately 17 kDa [22]. A recent reinvestigation revealed that the SSA polypeptide is not glycosylated and has a molecular mass of 16,618±2 Da [23]. In addition an N-terminal sequence of 20 amino acid residues was determined. This N-terminal sequence shares a high sequence identity with the N-terminus of a lectin from fruiting bodies of *Ciborinia camelliae* (CCL) [45] indicating that both closely related fungi express a similar lectin. However, at that time screening of the publicly accessible databases using the N-terminus of SSA and CCL as a query yielded no positive hits indicating that SSA and CCL are not related to any known protein, expressed protein or hypothetical protein. Regular updating of the BLAST searches remained unsuccessful until upon release of the Genome Sequence of *S. sclerotiorum* 1980 in September 2005 a whole genome shotgun sequence was retrieved (gb|AAGT01000457.1| *S. sclerotiorum* 1980 cont1.457) comprising an almost perfect match with the N-terminal sequence of SSA. However, the genomic sequence did not comprise an uninterrupted open reading frame (ORF) corresponding to a polypeptide of the same length as SSA, indicating that the identified gene contains intron(s). Manual analysis allowed determining a tentative ORF by joining nucleotides (91622..91731, 91802..91923, 91979..92078, 92144..92273). This ORF encodes a 153 amino acid polypeptide that apart from the N-terminal Met residue has the same N-terminus as SSA (Fig. 2a) and has calculated amino acid composition very similar to that of SSA [22] (results not shown).

To check the correctness of the presumed ORF the corresponding cDNA was isolated and sequenced. cDNA prepared from developing sclerotes was amplified by PCR using primers complementary to the 5′ and 3′ end of the putative ORF identified in the genomic sequence. A single fragment of approximately 450 bp was amplified. Sequencing demonstrated that the PCR fragment was identical to the ORF spliced from the genomic sequence. Alignment of the genomic and cDNA sequences confirmed that the coding region consists of four exons and three introns (Fig. 2a).

According to the deduced sequence the *SSA* gene encodes a 153 amino acid residue primary translation product. Comparison to the N-terminal sequence of native SSA indicates that the N-terminal Met residue is post-translationally removed from the primary translation product. The calculated molecular mass of the resulting polypeptide (16,608.4 Da) is nearly identical to the value measured by mass spectrometry (16,618 Da) indicating that no further post-translational modifications take place. Hence the mature SSA subunit comprises 152 amino acid residues.

Carbohydrate-binding specificity of SSA

The sugar-binding specificity of SSA was studied in detail by frontal affinity chromatography. Since FAC allows measuring K_d values according to the basic equation $K_d = B_t/(V - V_0)$ [30–33], we first determined B_t values by concentration-dependent analysis with 2 *p*-Nitrophenyl (pNP)-glycosides, GalNAcα and Galβ1-3GalNAcα (Core1). B_t and K_d values were calculated from Woolf-Hofstee type plots. For Galβ1-3GalNAcα-pNP (Fig. 3a) the calculated B_t

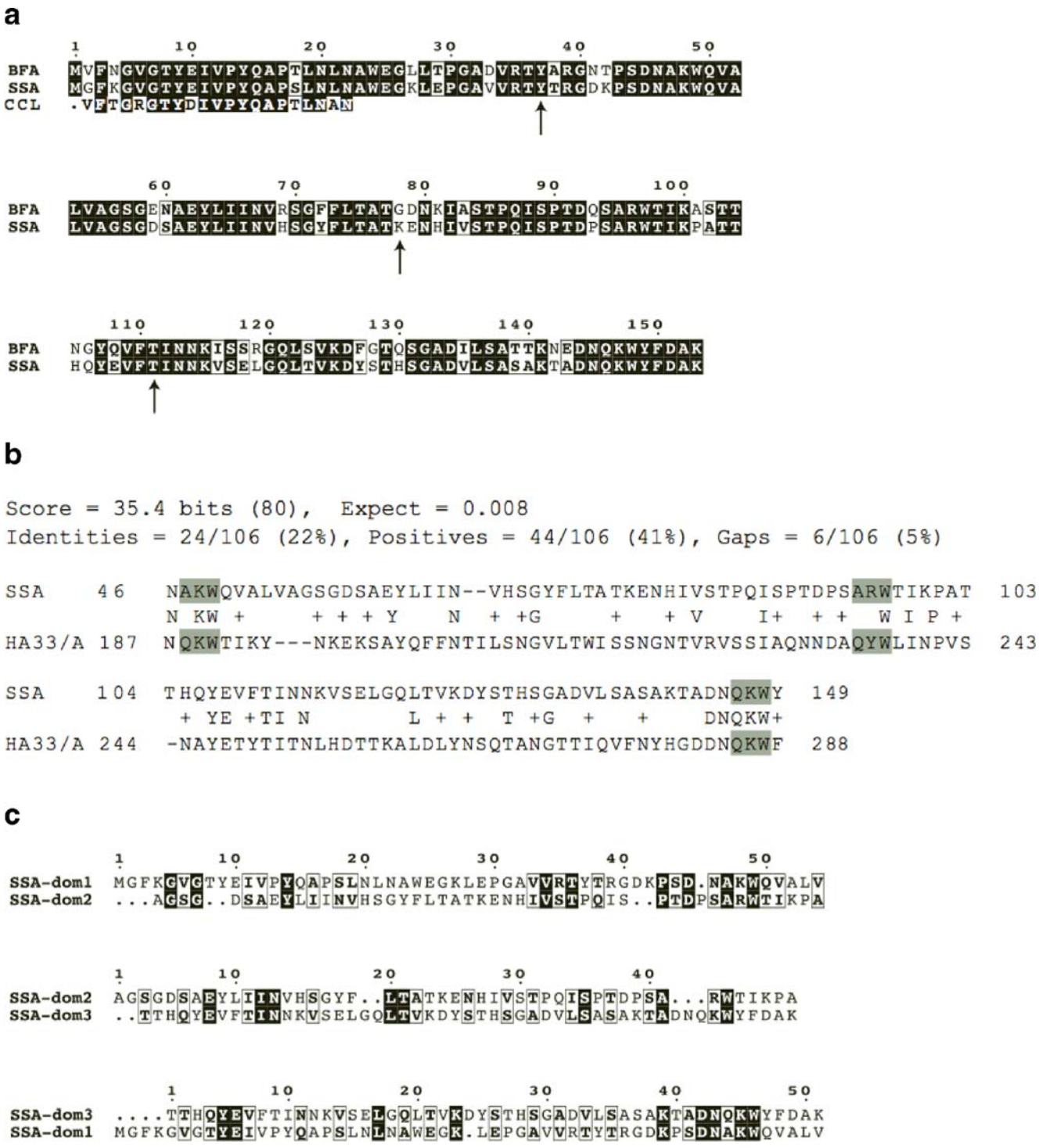

Fig. 2 **a** Alignment of the amino acid sequences of SSA and BFA, and the N-terminal sequence of CCL. Identical and homologous residues are *black boxed* and *open boxed*, respectively. The position of introns in the corresponding genomic sequences is indicated by *arrows*. **b** Graphic representation of the best (identified and characterized protein) hit of a PSI-Blast search [51] using SSA as a query. HA33/A (gi|870935|emb|CAA55714.1|) refers to the non-toxin haemagglutinin HA34 from *C. botulinum*. Putative QXW motifs are *boxed grey*. Note that the overlap covers only part of SSA and HA33/A. **c** Alignment of internal repeats in SSA. Results of CLUSTAL-X alignment were displayed with ESPript using the Risler's structural matrix for homologous residues. The sequence of BFA was deduced from gb|AAID01001952.1| *B. fuckeliana* B05.10 cont1.1952, whole genome shotgun sequence by joining complement (16735..16864, 16934..17033, 17093..17214, 17283..17392) and confirmed by the deduced sequences two of ESTs (gi|116086678|gb|EB807598.1| and gi|116078251|gb|EB801836.1|)

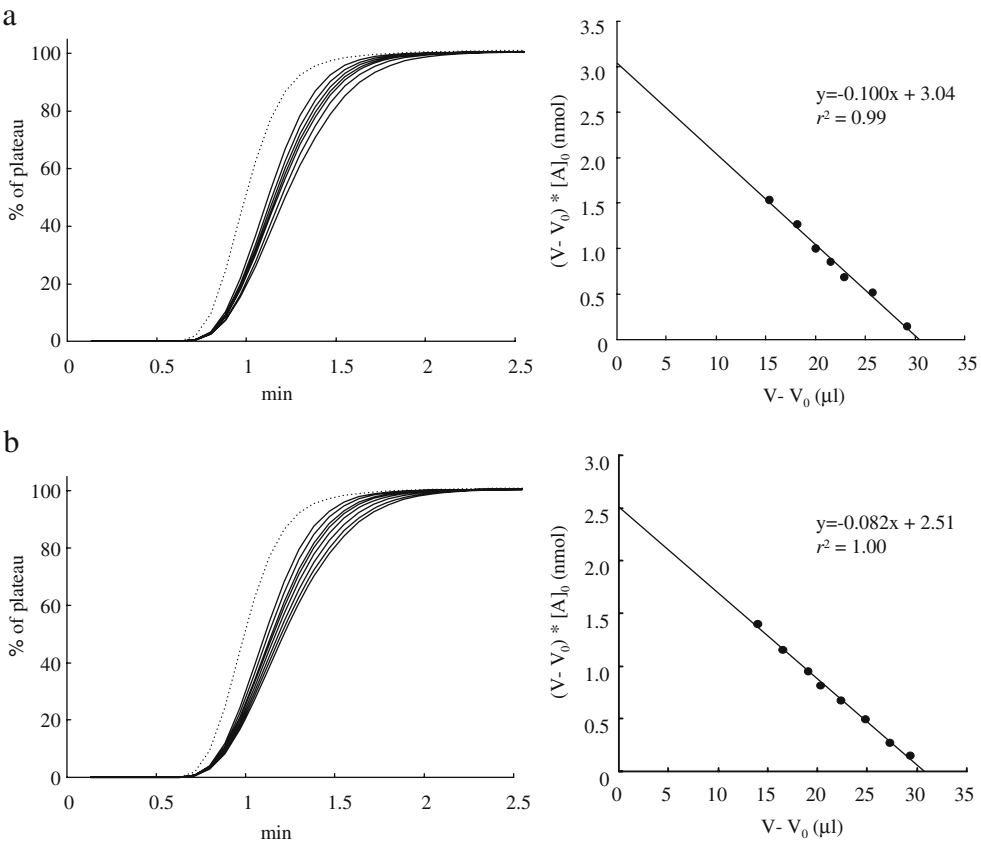

Fig. 3 Determination of the effective ligand content (B_t values) of the SSA-Sepharose FF column used in the FAC analysis. GalNAcα-pNP (**a**) and Galββ?1-3GalNAcα (Core1)-pNP (**b**) were diluted to various concentrations (5 to 100 μM) and applied to the SSA-agarose column. *Solid lines* represent elution profiles of GalNAcα- or Core1-pNP. Elution profiles of the control sugar (Fucα-pNP) are indicated by *dotted lines*. Panels on the *right* show the corresponding Woolf-Hofstee type plots generated from the $V-V_0$ values

and K_d values were 2.51 nmol and 8.2×10^{-5} M, respectively, and for GalNAcα-pNP (Fig. 3b) 3.04 nmol and 1.0×10^{-4} M (Fig. 3b), respectively. Accordingly, an averaged B_t value of 2.78 nmol was considered optimal for determination of K_d values for a series of other pNP-sugars as well as pyridylaminated (PA)-glycans.

The results of the K_d value measurements with a set of 18 pNP-sugars (summarized in Table 1) revealed that SSA apparently specifically recognizes galactose as a monosaccharide. Substitution of the C2 hydroxyl by an *N*-acetyl group substantially enhanced the affinity. SSA exhibited a slight preference for α- over β-anomers of both Gal-pNP and GalNAc-pNP. None of the tested pNP-oligosaccharides proved to be a substantially better ligand than GalNAc-pNP indicating that the binding site essentially accommodates a monosaccharide. It should be mentioned here that the use of *p*-Nitrophenyl (pNP) glycosides can influence the binding properties of some lectins through an interaction between hydrophobic sites adjacent to the binding site and aromatic aglycones of sugar derivatives. However, taking into account that neither *p*-nitrophenyl nor methyl-substituted α/β-D-galactopyranoside were better inhibitors of the interaction between SSA and asialofetuin than α/β-D-galactopyranoside itself (in a surface plasmon resonance based assay [23]), it is unlikely that the sugar binding activity of SSA is influenced by the reporter group of the sugars derivatives listed in Table 1.

FAC analysis with PA-glycans confirmed the results obtained with the pNP-sugars (Fig. 4, Table 2). Though the observed affinity was lower than that measured for

Table 1 K_d values of the interaction between SSA and *p*-nitrophenyl (pNP)-sugars as determined by frontal affinity chromatography

pNp-sugars	$K_d\times10^4$(M)
Galα-pNP	2.8
Galβ-pNP	3.3
GalNAcα-pNP (Tn)	1.0[a]
GalNAcβ-pNP	1.6
Manα-pNP	–
Manβ-pNP	–
Glcα-pNP	–
Glcβ-pNP	–
GlcNAcα-pNP	–
GlcNAcβ-pNP	–
Fucα-pNP	–
Lacβ-pNP	8.6
LacNAcβ-pNP	6.2
Forssman penta-pNP	6.5
GAlβ1-3GalNAcα-pNP (Core1, T)	0.82[a]
GlcNAcβ1-6(Galβ1-3)GalNAcα-pNP (Core2)	1.9
GlcNAcβ1-3GalNAcα-pNP (Core3)	–
GlcNAcβ1-6GalNAcα-pNP (Core6)	–

[a] K_d and K_a values were directly determined by concentration-dependence analysis.
–, no apparent binding was observed.

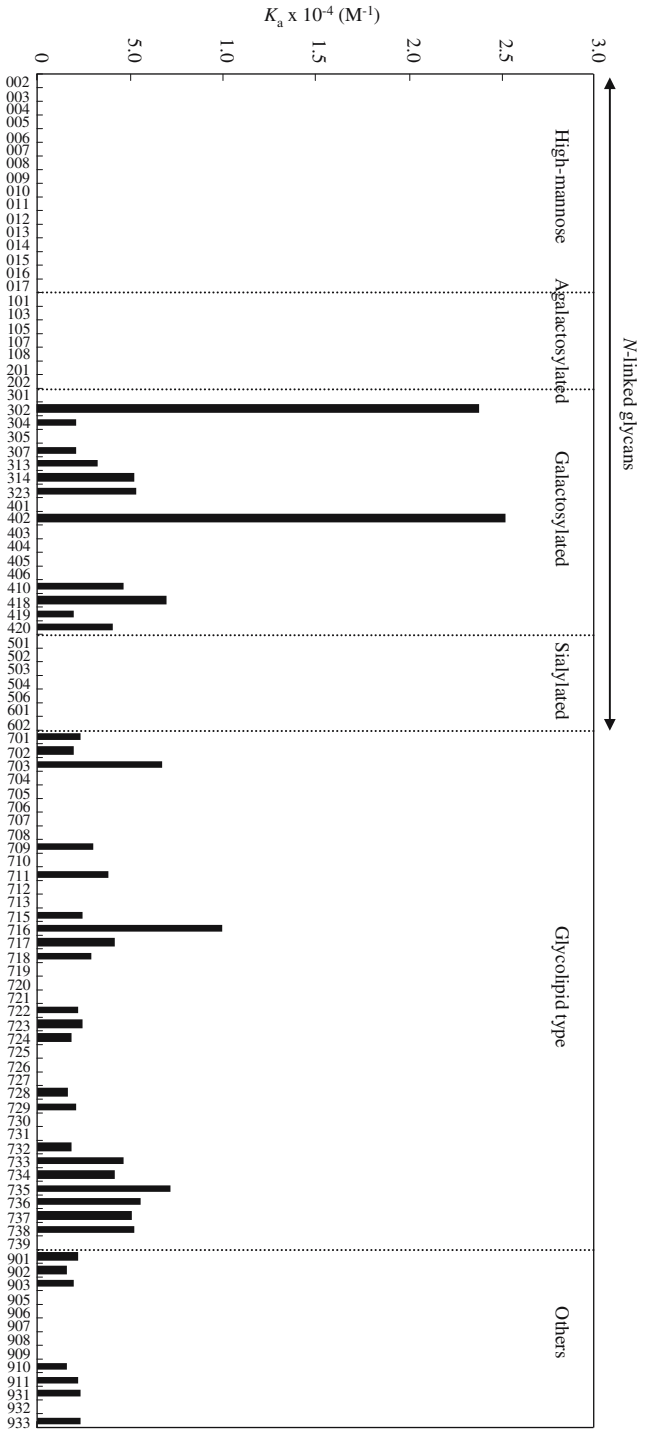

Fig. 4 Graphical representation of the affinity constants (K_a) of the interaction between SSA and the different glycans included in the FAC analysis. Arabic figures at the *bottom* of the graph correspond to number of the glycans shown in Fig. 1. The major classes of glycans are indicated at the *top* of the graph

Galβ1-3GalNAc*α-pNP* $\left(K_d = 8.2 \times 10^{-5}\mathrm{M}\right)$, SSA definitely interacted with Galβ1-3GalNAc-PA and other Galβ1-3GalNAc-containing glycans. A first group of reactive compounds include glycolipid-type glycans, **703** (GA1; $K_d = 1.5 \times 10^{-4}$M), **709** (GM1; 3.5×10^{-4} M) and **711** (GD1b; 2.7×10^{-4} M). Substituting 3-OH of Gal with NeuAc (as in **710, 712** and **713**) completely abolished the binding activity, which confirms the above mentioned conclusion that SSA requires unsubstituted non-reducing terminal Gal/GalNAc residues for binding activity. Several other glycans with either terminal non-reducing β1-3Gal/GalNAc (**e.g. 716, 728, 732, 733, 735, 736, 737, 738** and **911)** or terminal non-reducing β1-4Gal/GalNAc (**e.g. 701, 702, 724, 733, 734, 901, 902** and **903)** were found to interact with SSA (Fig. 4, Table 2). Among them, **716** (Gb4) with terminal β1-3GalNAc $\left(K_d = 1.0 \times 10^{-4}\mathrm{M}\right)$ was the best ligand for SSA. A series of βGalLac-containing glycans (**735, 736, 737** and **738**) was also recognized by SSA. The number of βGal units did not affect the affinity of SSA, which again confirms that the binding site of SSA accommodates only a single non-reducing terminal residue. LNT (**728**) and LNnT (**724**), which represent lacto- and neolacto-series glycolipids, respectively, also interacted with SSA. However, substitution of these glycans by αFuc (blood group H; **729**, Lewis^a; **730**, Lewis^b; **731**, Lewis^x; **726**), and αFuc combined with αGalNAc (blood group A; **720** and **721**) or αGal residues (blood group B; **739**) abolished their reactivity. The inability of Lewis^a (**730**) and Lewis^x (**726**) to interact with SSA, which is rather unexpected, suggests that the presence of Lewis-type Fuc prevents binding by SSA most probably through steric hindrance.

FAC analysis with N-linked glycans revealed that some galactosylated complex-type glycans were good ligands for SSA, whereas high-mannose type as well as agalactosylated complex-type glycans failed to interact with the lectin (Fig. 4, Table 2). Among the galactosylated N-linked glycans, SSA had the highest affinity for the α1-3 branched mono-antennary glycans **302** $\left(K_d = 4.2 \times 10^{-5}\mathrm{M}\right)$ and **402** (4.0×10^{-5} M). It should be noted here that the affinity of SSA for **302** and **402** exceeds that for the most reactive pNP sugar (in casu Core1-pNP). Surprisingly, SSA did not react with the α1-6 branched mono-antennary glycans **301** and **401**, which are isomers of the highly reactive structures **302** and **402**, respectively. This implies that the occurrence in N-glycans of a terminal galactose is required but not sufficient for recognition by SSA. Binding apparently also depends on the branching features of the N-glycans. At present, the mechanism underlying the branching specific binding is not understood.

Besides mono-antennary glycans, SSA also reacts with di and multi-antennary galactosylated N-glycans. Though the affinity apparently increased as a function of branch numbers (bi-antennary **307**, $K_d = 5.1 \times 10^{-4}$M; tri-antennary **313**, 3.2×10^{-4} M; tetra-antennary **323**, 1.9×10^{-4} M) the interaction was much weaker than with the mono-antennary structure **302**. Comparison of the tri-antennary linkage

Table 2 K_d values determined by frontal affinity chromatography pyridylaminated (PA)-glycans and SSA

$K_d \times 10^4$(M)							
PA-glycans		PA-glycans		PA-glycans		PA-glycans	
002	–	304	5.2	701	4.5	729	5.0
003	–	305	–	702	5.6	730	–
004	–	307	5.1	703	1.5	731	–
005	–	313	3.2	704	–	732	6.1
006	–	314	2.0	705	–	733	2.2
007	–	323	1.9	706	–	734	2.5
008	–			707	–	735	1.4
009	–	401	–	708	–	736	1.8
010	–	402	0.40	709	3.5	737	2.0
011	–	403	–	710	–	738	2.0
012	–	404	–	711	2.7	739	–
013	–	405	–	712	–		
014	–	406	–	713	–	901	4.9
015	–	410	2.2	715	4.3	902	6.8
016	–	418	1.5	716	1.0	903	5.5
017	–	419	5.3	717	2.5	905	–
		420	2.6	718	3.6	906	–
101	–			719	–	907	–
103	–	501	–	720	–	908	–
105	–	502	–	721	–	909	–
107	–	503	–	722	4.8	910	6.6
108	–	504	–	723	4.3	911	4.7
201	–	506	–	724	5.9	931	4.6
202	–			725	–	932	–
		601	–	726	–	933	4.7
301	–	602	–	727	–		
302	0.42			728	6.7		

The numbers of PA-glycans are corresponding to those indicated in Fig. 1.
–, no apparent binding was observed.

isomers further indicated that SSA has a 1.6 times higher affinity for the β1-3Gal structure **314** (2.0×10^{-4} M) than for the β1,4Gal structure **313**$\left(K_d = 3.2 \times 10^{-4}M\right)$. Addition of bisecting GlcNAc (**304** vs. **305**), α1-3 fucosylation (**313** vs. **419**, and **323** vs. **420**) and α2-6 sialylation (**307** vs. **503**, and **313** vs. **504**) dramatically reduced the binding affinity whereas α1-6 fucosylation of GlcNAc (**313** vs. **410,** and **323** vs. **418**) had a slight positive effect.

'Microarray screening experiments by the Consortium for Functional Glycomics' confirmed the results of the FAC analysis. The results of these screening experiments, which have recently been made publicly accessible by the consortium (http://www.functionalglycomics.org/glycomics/publicdata/primaryscreen.jsp) also clearly indicated that the specificity of SSA is primarily directed against Gal and GalNAc with a slight preference for the α- over the β-anomers. Some oligosaccharides (e.g. Galβ1-3GalNAcα1 and GalNAcβ1-3(Fucα1-2)Galβ) react slightly better with SSA than GalNAcα. However, the observed differences are not indicative for the presence of a complex carbohydrate-binding site.

SSA represents a novel family of fungal lectins

BLASTp and PSI-BLAST/PHI-BLAST searches of fungal databases using the sequence of SSA as a query did not yield any positive hit indicating that the lectin shares no detectable sequence similarity with any other sequenced or cloned fungal protein and accordingly represents a novel family of fungal lectins. Upon extending the searches to cDNA, EST and genomic databases no other expressed or hypothetical protein with a detectable sequence similarity to the SSA could be identified except in *Botryotinia fuckeliana*. A whole genome shotgun sequence (gb|AAID01001952.1| *B. fuckeliana* B05.10 cont1.1952) of this fungus, which is classified in the same family as *Sclerotinia*, contains a gene that can be considered a genuine orthologue of the *SSA* gene because it has the

same exon/intron structure and comprises an ORF encoding a protein that shares 77.12 and 94.11% sequence identity and similarity, respectively, with SSA (Fig. 2a). Recently, a transcriptome analysis of *B. fuckeliana* yielded two ESTs from a mycelial cDNA library (gi|116086678|gb|EB807598.1| and gi|116078251|gb|EB801836.1|) that perfectly match the amino acid sequence inferred from the *B. fuckeliana* agglutinin gene *(BFA)* [46] leaving no doubt that this gene is expressed and most probably encodes the previously described lectin from the same species (formerly referred to as *Botrytis cinerea* agglutinin or BCA) [22].

Molecular modeling predicts that SSA is structurally related to the ricin-B superfamily

BLASTp and PSI-BLAST/PHI-BLAST searches using the sequences of SSA and BFA as queries yielded no genuine homologues but revealed a residual sequence identity of approximately 20% with part of the non-toxin haemagglutinin HA33/A from *C. botulinum* (Fig. 2b). HA33, which is associated with the large botulinum neurotoxin secreted complexes exhibits a unique domain organization and possesses a carbohydrate recognition site located in a ricin-type β-trefoil domain. Since the latter domain is a common structural unit of many diverse carbohydrate-binding proteins from both prokaryotes and eukaryotes the residual similarity between SSA/BFA and the sugar-binding domain of HA33/A raised the question whether the Sclerotiniaceae lectins possibly belong to ricin-B superfamily.

A closer examination of the sequences revealed two features of SSA/BFA that are reminiscent to the ricin-B domain. First, the sequences contain a typical QXW motif at their C-terminus. Moreover, the presence in SSA/BFA of two W residues at the same position as the tryptophane of the first and second QXW motifs in the overlapping domain of HA33/A (Fig. 2b) suggest the occurrence of two additional less conserved QXW motifs in the fungal lectins. Secondly, the lectin polypeptides consist of three subdomains with a residual internal homology. This residual internal homology is not recognized upon aligning all three presumed subdomains but becomes clear when the subdomains are pairwise aligned (Fig. 2c). To further corroborate the possible structural relationship with the ricin-B domain an attempt was made to predict the overall fold of SSA/BFA by molecular modeling. In spite of the low residual identity and similarity between SSA/BFA and HA33/A, HCA revealed a markedly similar overall fold for both proteins (Fig. 5). Due to this apparently conserved fold fairly accurate models could be built of the three-dimensional structure of SSA and BFA using the atomic coordinates of 1YBI as a template. As shown in Fig. 6, the subunits of SSA and BFA are folded into a β-trefoil domain consisting of six pairs of antiparallel strands of β-sheet connected by extended loops. The twelve strands of β-sheet are arranged around a pseudo 3-fold axis into three four-stranded β-trefoil repeats interconnected by loops. This all-β organization is in perfect agreement with previous far-UV circular dichroism spectra showing that SSA is predominantly built from β-sheet secondary structures and contains no α-helical regions [23]. Assuming that the predicted structural homology between SSA/BFA and the ricin-B domain also applies to the binding site(s) a putative sugar-binding site might be located in SSA/BFA in the N-terminal region corresponding to subdomain 1α of the ricin-B. Though purely speculative, the appearance of this subdomain on the surface of the protein as a shallow depression with a rather pronounced electronegative char-

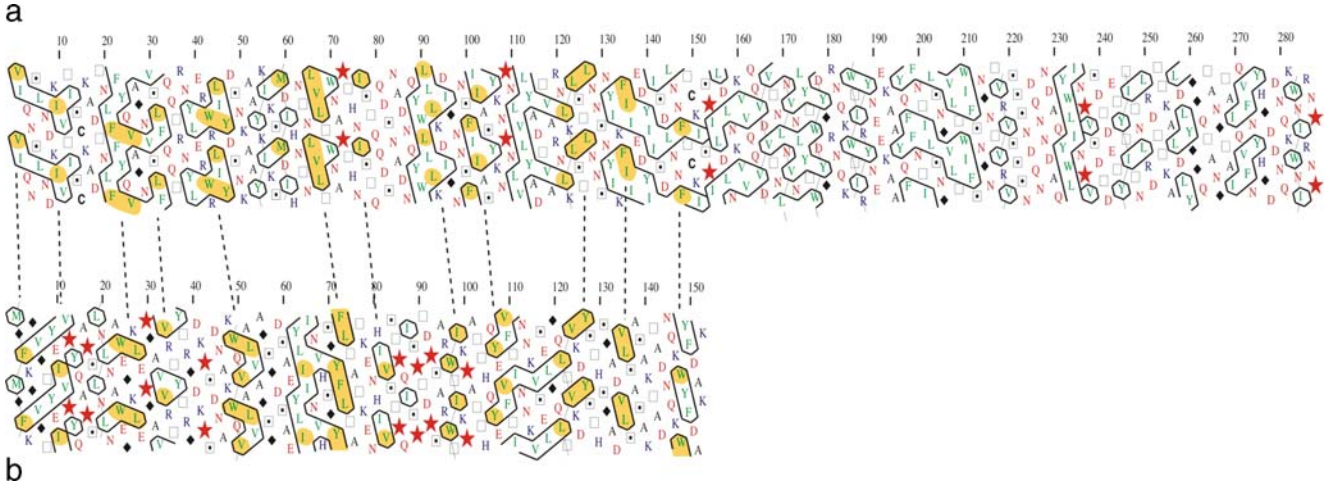

Fig. 5 Hydrophobic cluster analysis of the non-toxin haemagglutinin HA33/A from *C. botulinum* (**a**) and SSA (**b**). Corresponding (*dashed lines*) hydrophobic residues forming the hydrophobic clusters are colored orange

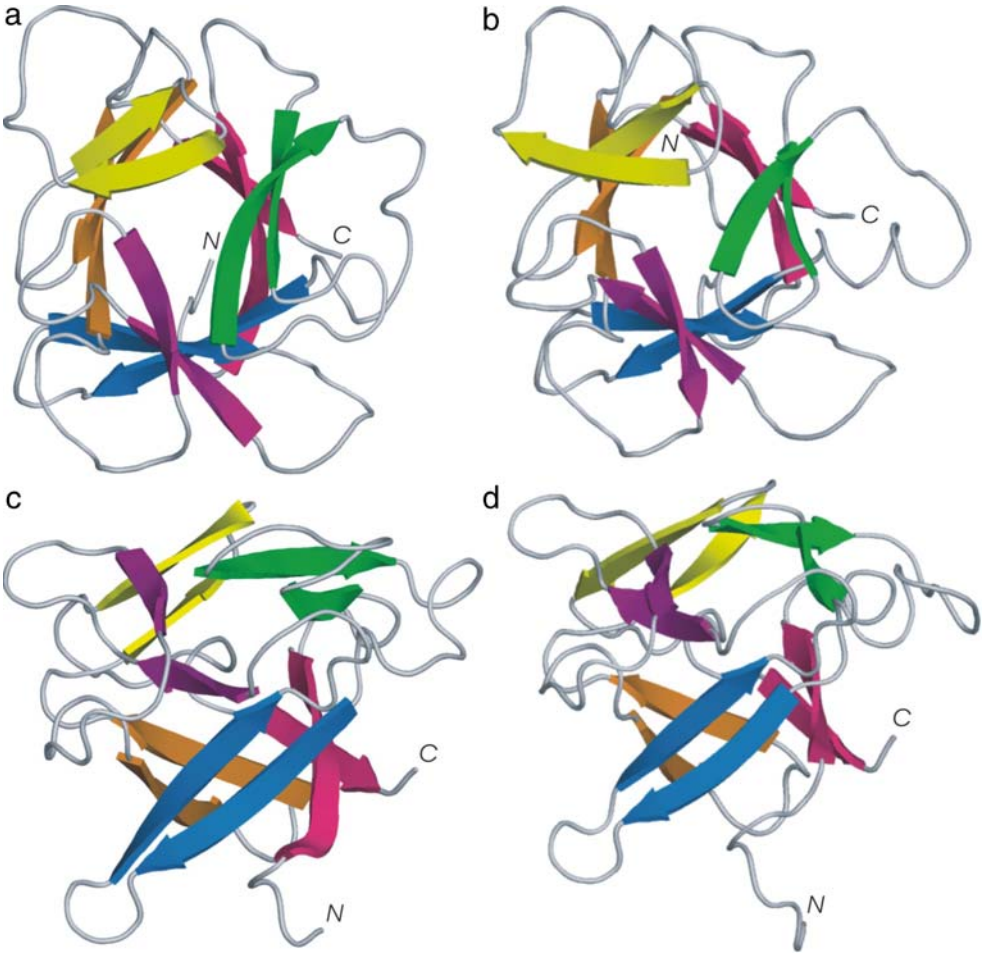

Fig. 6 *Ribbon diagrams* of the overall fold of SSA and BFA. **a** and **b** represent *frontal views* of SSA and BFA, respectively. *Sagital views* of SSA and BFA are shown in **c** and **d**, respectively. The six pairs of antiparallel β-strands forming the β-trefoil fold are shown in *pink, green, blue, magenta, orange* and *yellow*, respectively. *N* and *C* correspond to the N- and C-terminal ends of the lectin domains, respectively

acter (result not shown) is reminiscent to that of many other carbohydrate-binding sites.

Discussion

The occurrence of an agglutinin in the phytopathogenic fungus *Sclerotinia sclerotiorum* was for the first time reported in a comparative study of lectins from different members of the family Sclerotiniaceae [22]. Biochemical analyses demonstrated that *Sclerotinia* species as well as *Botryotinia fuckeliana* express lectins that have the same molecular structure and a very similar amino acid composition, and exhibit a similar specificity towards Gal/GalNAc. In addition all studied lectins were closely related serologically, indicating that the Sclerotiniaceae lectins represent a homogeneous group of fungal lectins. More detailed biochemical analyses [23] yielded an accurate value for the molecular mass of the subunits (16,618 Da) whereas CD spectra analysis indicated that SSA contains predominantly β-sheet structures. Furthermore, N-terminal sequencing revealed that SSA shares no sequence similarity with any other protein except a lectin from another Sclerotiniaceae species *C. camelliae* [45]. These novel findings confirmed that the Sclerotiniaceae lectins are a homogeneous family of lectins and provided preliminary evidence that they might be unrelated to any other family of fungal lectins.

To answer the question whether the Sclerotiniaceae lectins really represent a distinct family of proteins the gene encoding SSA was retrieved from the genome of *S. sclerotiorum* and the corresponding cDNA cloned. The results of this approach unambiguously demonstrated that SSA shares no sequence similarity with any known fungal lectin or protein and hence represents a novel family of fungal lectins. Alignment of the cDNA and genomic sequences demonstrated that the coding sequence of the *SSA* gene comprises three introns. The ORF encodes a 153 amino acid residue polypeptide that apparently lacks a signal peptide suggesting that the lectin is synthesized on free ribosomes. Comparison to the mature lectin polypeptide indicates that the primary translation product undergoes apart from the removal of the N-terminal methionine no further processing.

Using the cDNA sequence of SSA as a query an orthologue of the *SSA* gene could readily be identified in the genome of *B. fuckeliana*. The *BFA* gene has the same exon/intron structure as the *SSA* gene and contains an ORF

encoding a polypeptide that apparently corresponds to the previously described *B. cinerea* agglutinin. Besides SSA, lectins with a virtually identical molecular structure and amino acid composition have been isolated from *S. minor, S. miyabaena* and *S. trifoliorum* [22]. In addition, it seems very likely that also the lectin isolated from *C. camellia* [45], which is another member of the Sclerotiniaceae, can be considered a genuine orthologue of SSA because it has the same molecular structure and amino acid composition, and shares a high sequence identity with SSA at its N-terminus. Based on these observations it can be concluded that SSA-type lectins are apparently fairly common within the family Sclerotiniaceae. Moreover, even though no definitive conclusions can be drawn on the basis of the available sequence data, the apparent absence from all (nearly) completed fungal genomes strongly suggests that genes encoding proteins similar to SSA and BFA are confined to a small taxonomic group of the Ascomycota. This narrow taxonomic distribution might in turn indicate that the SSA-type protein family evolved after the ancestor of modern Sclerotiniaceae diverged from other Ascomycota. However, if so, the apparent absence of even distantly related genes from other Ascomyta poses a problem for what concerns the origin of the ancestor of the Sclerotiniaceae lectin genes.

PSI-BLAST/PHI-BLAST searches revealed a residual sequence similarity between SSA and a ricin-type β-trefoil domain found in the non-toxin haemagglutinin HA33/A from *C. botulinum* [38]. Molecular modeling using the three-dimensional structure of HA33/A as a template confirmed that SSA can fold into a similar β-trefoil domain. Though it should be emphasized that modelling has only a predictive value, the results obtained by this approach indicate that the Sclerotiniaceae lectins are structurally related to the ricin-B superfamily. However, the low sequence similarity to HA33/A and especially the lack of detectable similarity with any other fungal, plant, animal or bacterial ricin-B type carbohydrate-binding unit make it questionable whether SSA is also evolutionary related to the ricin-B domain. Structural studies are in progress to address this issue.

Previous studies using hapten inhibition assays of the agglutination of animal erythrocytes and surface plasmon resonance indicated that SSA recognizes both Gal and GalNAc but provided no information about the affinity of the lectin for these sugars. Moreover, there were some discrepancies between the results obtained by both methods. To revisit the specificity of SSA detailed analyses were made by FAC. Measuring direct interactions between SSA and glycans allowed drawing some general conclusions with respect to the sugar binding specificity of SSA. First, the binding site of SSA primarily accommodates a non-reducing terminal GalNAc with a preference for the α- over the β-anomer. Non-reducing terminal Gal residues are also bound but with a lower affinity. Substitution of GalNAc by a β-1,3 linked Gal only slightly increases the affinity, suggesting that the binding site of SSA essentially corresponds to a monosaccharide binding site. Secondly, SSA strongly interacts with glycolipid type glycans with terminal non-reducing Gal or GalNAc but fails to bind sialylated or fucosylated forms of the same glycans. Third, SSA strongly interacts with galactosylated N-glycans. The lectin has the highest affinity for α1-3 branched mono-antennary N-glycans but also binds to multi-antennary glycans. Binding of SSA to N-glycans requires besides the presence of terminal non-reducing Gal some specific but yet undefined branching features. Though it is difficult to compare results from specificity studies obtained by different methods it appears that none of the previously studied fungal lectins exhibit the same fine specificity as SSA. This indicates that SSA distinguishes itself also with respect to its sugar-binding properties from all other fungal lectins.

The results of the molecular cloning and specificity studies urge to readress the question of the potential role of SSA and related Sclerotiniaceae lectins. At present, the role of lectins expressed by phytopathogenic fungi is poorly understood. A possible involvement in pathogenicity is unlikely because none of the identified fungal lectins exhibits a specificity towards typical plant glycans. Moreover, in the case of SSA and related lectins a role in pathogenesis is virtually excluded because they are synthesized in the cytoplasm and hence located on the 'wrong side' of the membrane. In this respect, SSA is reminiscent to a previously described lectin that accumulates in large quantities in the cytoplasm of mycelium and trap cells of the nematophagous fungus *Arthrobotrys oligospora*. Initially the *A. oligospora* lectin (AOL), which belongs to the family of fungal *A. bisporus* type agglutinins and specifically recognizes the T-antigen structure (Galβ1-3GalNAcα-Ser/Thr) was believed to be involved in nematode trapping [47]. However, later studies ruled out the role in nematode trapping [48] and provided firm evidence that AOL behaves as a typical cytoplasmic storage protein [49]. Taking into account the high expression level in sclerotes [47] SSA and other Sclerotinaceae lectins can certainly be considered functional homologues of AOL. Moreover, on the analogy of the dual storage-defence role attributed to a large group of abundant plant lectins [50] the Sclerotiniaceae lectins also might combine a likely function as a storage protein with a defensive role against possible predating organisms. According to this concept some Sclerotiniaceae species accumulate in their storage/surviving tissues large quantities of cytoplasmic storage proteins that by virtue of their sugar-binding properties are toxic or repellant for potential

predators. This implies that as long as the sclerotes are not challenged the lectins just act as a store of readily available nitrogen. When the sclerotes, which are destined to ensure the survival of the species, are attacked by either invertebrates or higher animals the lectins are released into the digestive tract where they interact with the glycans exposed on the surface of the epithelial cells and cause harmful effects that eventually repel the predators.

Acknowledgements This research was supported by the Fund for Scientific Research-Flanders, the Research Council of Ghent University and by NEDO (New Energy and Industrial Technology Organization) under the METI (The Ministry of Economy, Trade, and Industry, Japan).2

References

1. Dodd, R.B., Drickamer, K.: Lectin-like proteins in model organisms: implications for evolution of carbohydrate-binding activity. Glycobiology **11**, 71R–79R (2001)
2. Van Damme, E.J.M., Peumans, W.J., Barre, A., Rougé, P.: Plant lectins: a composite of several distinct families of structurally and evolutionary related proteins with diverse biological roles. Crit. Rev. Plant Sci. **17**, 575–692 (1998)
3. Carrizo, M.E., Capaldi, S., Perduca, M., Irazoqui, F.J., Nores, G. A., Monaco, H.L.: The antineoplastic lectin of the common edible mushroom (*Agaricus bisporus*) has two binding sites, each specific for a different configuration at a single epimeric hydroxyl. J. Biol. Chem. **280**, 10614–10623 (2004)
4. Birck, C., Damian, L., Marty-Detraves, C., Lougarre, A., Schulze-Briese, C., Koehl, P., Fournier, D., Paquereau, L., Samama, J.P.: A new lectin family with structure similarity to actinoporins revealed by the crystal structure of *Xerocomus chrysenteron* lectin XCL. J. Mol. Biol. **344**, 1409–1420 (2004)
5. Wimmerova, M., Mitchell, E., Sanchez, J.F., Gautier, C., Imberty, A.: Crystal structure of fungal lectin: six-bladed β-propeller fold and novel fucose recognition mode for *Aleuria aurantia* lectin. J. Biol. Chem. **278**, 27059–27067 (2003)
6. Cooper, D.N., Boulianne, R.P., Charlton, S., Farrell, E.M., Sucher, A., Lu, B.C.: Fungal galectins, sequence and specificity of two isolectins from *Coprinus cinereus*. J. Biol. Chem. **272**, 1514–1521 (1997)
7. Yagi, F., Hiroyama, H., Kodama, S.: *Agrocybe cylindracea* lectin is a member of the galectin family. Glycoconj. J. **18**, 745–749 (2001)
8. Kruger, R.P., Winter, H.C., Simonson-Leff, N., Stuckey, J.A., Goldstein, I.J., Dixon, J.E.: Cloning, expression, and characterization of the Galα1,3Gal high affinity lectin from the mushroom *Marasmius oreades*. J. Biol. Chem. **277**, 15002–15005 (2002)
9. Tateno, H., Winter, H.C., Goldstein, I.J.: Cloning, expression in *Escherichia coli* and characterization of the recombinant Neu5-Acα2,6Galβ1,4GlcNAc-specific high-affinity lectin and its mutants from the mushroom *Polyporus squamosus*. Biochem. J. **382**, 667–675 (2004)
10. Tateno, H., Goldstein, I.J.: Molecular cloning, expression, and characterization of novel hemolytic lectins from the mushroom *Laetiporus sulphureus*, which show homology to bacterial toxins. J. Biol. Chem. **278**, 40455–40463 (2003)
11. Mancheno, J.M., Tateno, H., Goldstein, I.J., Martinez-Ripoll, M., Hermoso, J.A.: Structural analysis of the *Laetiporus sulphureus* hemolytic pore-forming lectin in complex with sugars. J. Biol. Chem. **280**, 17251–17259 (2005)
12. Sumisa, F., Iijima, N., Ando, A., Shiga, M., Kondo, K., Amano, K., Nagata, Y.: Properties of mycelial aggregate-specific lectin of *Pleurotus cornucopiae* produced in *Pichia pastoris*. Biosci. Biotechnol. Biochem. **68**, 959–960 (2004)
13. Nagata Y., Yamashita M., Honda H., Akabane J., Uehara K., Saito A., Sumisa F., Nishibori K, Oodaira Y.: Characterization, occurrence, and molecular cloning of a lectin from *Grifola frondosa*: jacalin-related lectin of fungal origin. Biosci. Biotechnol. Biochem. **69**, 2374–2380 (2005)
14. Paaventhan, P., Joseph, J.S., Seow, S.V., Vaday, S., Robinson, H., Chua, K.Y., Kolatkar P.R.: A 1.7 Å structure of Fve, a member of the new fungal immunomodulatory protein family. J. Mol. Biol. **332**, 461–470 (2003)
15. Oda, Y., Senaha, T., Matsuno, Y., Nakajima, K., Naka, R., Kinoshita, M., Honda, E., Furuta, I., Kakehi, K.: A new fungal lectin recognizing α(1-6)-linked fucose in the N-glycan. J. Biol. Chem. **278**, 32439–32447 (2003)
16. Cioci, G., Mitchell, E.P., Chazalet, V., Debray, H., Oscarson, S., Lahmann, M., Gautier, C., Breton, C., Perez, S.: Imberty A, β-Propeller crystal structure of *Psathyrella velutina* lectin: an integrin-like fungal protein interacting with monosaccharides and calcium. J. Mol. Biol. **357**, 1575–1591 (2006)
17. Brown, D.W., Cheung, F., Proctor, R.H., Butchko, R.A., Zheng, L., Lee, Y., Utterback, T., Smith, S., Feldblyum, T., Glenn, A.E., Plattner, R.D., Kendra, D.F., Town, C.D., Whitelaw, C.A.: Comparative analysis of 87,000 expressed sequence tags from the fumonisin-producing fungus *Fusarium verticillioides*. Fungal. Genet. Biol. **42**, 848–861 (2005)
18. Machida, M., Asai, K., Sano, M., Tanaka, T., Kumagai, T., Terai, G., Kusumoto, K., Arima, T., Akita, O., Kashiwagi, Y., Abe, K., Gomi, K., Horiuchi, H., Kitamoto, K., Kobayashi, T., Takeuchi, M., Denning, D.W., Galagan, J.E., Nierman, W.C., Yu, J., Archer, D.B., Bennett, J.W., Bhatnagar, D., Cleveland, T.E., Fedorova, N.D., Gotoh, O., Horikawa, H., Hosoyama, A., Ichinomiya, M., Igarashi, R., Iwashita, K., Juvvadi, P.R., Kato, M., Kato, Y., Kin, T., Kokubun, A., Maeda, H., Maeyama, N., Maruyama, J., Nagasaki, H., Nakajima, T., Oda, K., Okada, K., Paulsen, I., Sakamoto, K., Sawano, T., Takahashi, M., Takase, K., Terabayashi, Y., Wortman, J.R., Yamada, O., Yamagata, Y., Anazawa, H., Hata, Y., Koide, Y., Komori, T., Koyama, Y., Minetoki, T., Suharnan, S., Tanaka, A., Isono, K., Kuhara, S., Ogasawara, N., Kikuchi, H.: Genome sequencing and analysis of *Aspergillus oryzae*. Nature **438**, 1157–1161 (2005)
19. Coltri, K.C., Casabona-Fortunato, A.S., Gennari-Cardoso, M.L., Pinzan, C.F., Ruas, L.P., Mariano, V.S., Martinez, R., Rosa, J.C., Panunto-Castelo, A., Roque-Barreira, M.C.: Paracoccin, a GlcNAc-binding lectin from *Paracoccidioides brasiliensis*, binds to laminin and induces TNF-α production by macrophages. Microbes Infect. **8**, 704–713 (2006)
20. Ng, T.B., Lam, Y.W.: Isolation of a novel agglutinin with complex carbohydrate binding specificity from fresh fruiting bodies of the edible mushroom *Lyophyllum shimeiji*. Biochem. Biophys. Res. Commun. **290**, 563–568 (2002)
21. Wang, H., Ng, T.B., Liu, Q.: A novel lectin from the wild mushroom *Polyporus adusta*. Biochem. Biophys. Res. Commun. **307**, 535–539 (2003)
22. Kellens, J.T.C., Peumans W.J.: Lectins in different members of the Sclerotiniaceae. Mycol. Res. **96**, 495–502 (1992)
23. Candy, L., Van Damme, E.J.M., Peumans, W.J., Menu-Bouaouiche, L., Erard, M., Rougé, P.: Structural and functional characterization of the GalNAc/Gal-specific lectin from the phytopathogenic ascomycete *Sclerotinia sclerotiorum* (Lib.) de Bary. Biochem. Biophys. Res. Commun. **308**, 396–402 (2003)
24. Nakamura, S., Yagi, F., Totani, K., Ito, Y., Hirabayashi, J.: Comparative analysis of carbohydrate-binding properties of two

tandem repeat-type jacalin-related lectins, *Castanea crenata* agglutinin and *Cycas revoluta* leaf lectin. FEBS J. **272**, 2784–2799 (2005)
25. Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature **227**, 680–685 (1970)
26. Mierendorf, R.C., Pfeffer, D.: Direct sequencing of denatured plasmid DNA. Methods Enzymol. **152**, 556–562 (1987)
27. Sanger, F., Nicklen, S., Coulson, A.R.: DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U. S. A. **74**, 5463–5467 (1977)
28. Bradford, M.M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248–254 (1976)
29. Hirabayashi, J.: Lectin-based structural glycomics: glycoproteomics and glycan profiling. Glycoconj. J. **21**, 35–40 (2004)
30. Arata, Y., Hirabayashi, J., Kasai, K.: Application of reinforced frontal affinity chromatography and advanced processing procedure to the study of the binding property of a *Caenorhabditis elegans* galectin. J. Chromatogr. A **905**, 337–343 (2001)
31. Kasai, K., Oda, Y., Nishikawa, M., Ishii, S.: Frontal affinity chromatography: theory, for its application to studies on specific interaction of biomolecules. J. Chromatogr. A **379**, 33–47 (1986)
32. Hirabayashi, J., Hashidate, T., Arata, Y., Nishi, N., Nakamura, T., Hirashima, M., Urashima, T., Oka, T., Futai, M., Muller, W.E., Yagi, F., Kasai, K.: Oligosaccharide specificity of galectins: a search by frontal affinity chromatography. Biochim. Biophys. Acta **1572**, 232–254 (2002)
33. Hirabayashi, J., Arata, Y., Kasai, K.: Frontal affinity chromatography as a tool for elucidation of sugar recognition properties of lectins. Methods Enzymol. **362**, 352–368 (2003)
34. Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G.: The CLUSTAL-X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tool. Nucleic Acids Res. **15**, 4876–4882 (1997)
35. Gouet, P., Courcelle, E., Stuart, D.I., Metoz, F.: ESPript: analysis of multiple sequence alignments in PostScript. Bioinformatics **15**, 305–308 (1999)
36. Risler, J.-L., Delorme, M.O., Delacroix, H., Henaut, A.: Amino acid substitutions in structurally related proteins in pattern recognition approach. Determination of a new and efficient scoring matrix. J. Mol. Biol. **204**, 1019–1029 (1998)
37. Gaboriaud, C., Bissery, V., Benchetrit, T., Mornon, J.P.: Hydrophobic cluster analysis: an efficient new way to compare and analyse amino acid sequences. FEBS Lett. **224**, 149–155 (1987)
38. Arndt, J.W., Gu, J., Jaroszewski, L., Schwarzenbacher, R., Hanson, M.A., Lebeda, F.J., Stevens, R.C.: The structure of the neurotoxin-associated protein HA33/A from *Clostridium botulinum* suggests a reoccurring β-trefoil in the progenitor toxin complex. J. Mol. Biol. **346**, 1083–1093 (2005)
39. Ponder, J.W., Richards, F.M.: Tertiary templates for proteins. Use of packing criteria in the enumeration of allowed sequences for different structural classes. J. Mol. Biol. **193**, 775–791 (1987)
40. Mas, M.T., Smith, K.C., Yarmush, D.L., Aisaka, K., Fine, R.M.: Modeling the anti-CEA antibody combining site by homology and conformational search. Proteins Struc. Func. Genet. **14**, 483–498 (1992)
41. Roussel, A., Cambillau, C.: TURBO-FRODO, silicon graphics geometry partners directory. In: Committee S.G. (eds.). Mountain View, California, Silicon Graphics (1989)
42. Laskowski, R.A., MacArthur, M.W., Moss, D.S., Thornton, J.M.: PROCHECK: a program to check the stereochemistry of protein structures. J. Appl. Cryst. **26**, 283–291 (1993)
43. Nicholls, A., Sharp, K.A., Honig, B.: Protein folding and association: insights from the interfacial and thermodynamic properties of hydrocarbons. Proteins Struc. Func. Genet. **11**, 281–296 (1991)
44. Gilson, M.K., Honing, B.H.: Calculation of electrostatic potential in an enzyme active site. Nature **330**, 84–86 (1987)
45. Otta, Y., Amano, K., Nishiyama, K., Ando, A., Ogawa, S., Nagata, Y.: Purification and properties of a lectin from ascomycete mushroom, *Ciborinia camelliae*. Phytochemistry **60**, 103–107 (2002)
46. Silva, E., Valdes, J., Holmes, D., Shmaryahu, A., Valenzuela, P. D.: Generation and analysis of expressed sequence tags from *Botrytis cinerea*. Biol. Res. **39**, 367–376 (2006)
47. Nordbring-Hertz, B., Mattiasson, B.: Action of a nematode-trapping fungus shows lectin-mediated host-microorganism interaction. Nature **281**, 477–479 (1979)
48. Balogh, J., Tunlid, A., Rosen, S.: Deletion of a lectin gene does not affect the phenotype of the nematode-trapping fungus *Arthrobotrys oligospora*. Fungal Genet. Biol. **39**, 128–135 (2003)
49. Rosen, S., Sjollema, K., Veenhuis, M., Tunlid, A.: A cytoplasmic lectin produced by the fungus *Arthrobotrys oligospora* functions as a storage protein during saprophytic and parasitic growth. Microbiology **143**, 2593–2604 (1997)
50. Van Damme, E.J.M., Barre A., Rougé, P., Peumans, W.J.: Cytoplasmic/nuclear plant lectins: a new story. Trends Plant Sci. **9**, 484–489 (2004)
51. Schäffer, A.A., Aravind, L., Madden, T.L.: Shavirin, S., Spouge, J.L., Wolf, Y.I., Koonin, E.V., Altschul, S.F.: Improving the accuracy of PSI-BLAST protein database searches with composition-based statistics and other refinements. Nucleic Acids Res. **29**, 2994–3005 (2001)