Lectin blot studies on proteins of *Myxobolus cerebralis*, the causative agent of whirling disease

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ABSTRACT: It is known that *Myxobolus cerebralis* antigens, both surficial and secreted, are key modulators for, or targets of, host immune system compounds. We undertook SDS-PAGE glyco-protein characterisation of *M. cerebralis* developmental stages isolated from infected rainbow trout and Western blot analyses using selected biotin-labelled plant lectins (GSA-I, PHA-E, SJA, GSA-II) and anti-triactinomyxon polyclonal antibodies. Glycoproteins were isolated with lectin-affinity chro-matography, and prominent bands were characterised by matrix-assisted laser desorption/ionisation-mass spectrometry (MALDI/MS). We identified glycoproteins of *M. cerebralis* myxospores that con-tained carbohydrate motifs reactive with *Phaseolus vulgaris* erythroagglutinin (proteins 20 to 209 kDa, PHA-E), *Sophora japonica* agglutinin (proteins 7 to 70 kDa, SJA), *Griffonia simplicifolia* Agglutinin I (proteins 10 to 209 kDa, GSA-I) and *G. simplicifolia* Agglutinin II (proteins 5 to 40 kDa, GSA-II). Mcgp33, a glycoprotein isolated by lectin-affinity chromatography, was reactive with SJA (about 33 kDa). Antiserum produced against *M. cerebralis* triactinomyxons was found to have differ-ences in the antigenicity of isolated glycoproteins from both *M. cerebralis* myxospores and acti-nospores. We also demonstrated modified antigen expression, especially involving the glycoprotein Mcgp33, in different developmental stages of *M. cerebralis*.

KEY WORDS: *Myxobolus cerebralis* · Glycoprotein · Lectin · MALDI/MS · Lectin blotting · *Oncorynchus mykiss* · Myxozoa · Interaction

INTRODUCTION

Myxobolus cerebralis (Myxozoa, Hofer 1903), the causative agent of whirling disease in salmonid fish, has a 2-host life cycle (Wolf & Markiw 1984, El-Mat-bouli & Hoffmann 1989), with an oligochaete (*Tubifex tubifex*) as the definitive host (El-Matbouli & Hoffmann 1989, 1998) and a salmonid, in particular rainbow trout *Oncorhynchus mykiss*, as an intermediate host. Water-borne triactinomyxon spores (the actinospore stage) are released from infected oligochaetes, and remain suspended in the water column until they encounter a susceptible salmonid fish. This developmental stage is only infectious for susceptible fish species (El-Matbouli & Hoffmann 1998). After a rapid process of host recog-nition, the spores attach to the fish epidermis and release their infective sporoplasms (El-Matbouli et al. 1995). Sporoplasm cells penetrate the epidermis and migrate to the peripheral nerve system, through the medulla spinalis to their target tissues: cerebral and spinal cartilage. About 60 d after infection, following a period of proliferation, sporogenesis begins and gener-ates thousands of myxobolus spores (the myxospore stage). Myxospores are usually released from the host when the fish dies and decays, but can also remain infectious if the fish is consumed by a predator (El-Matbouli & Hoffmann 1991) and they are excreted in faeces; Nehring et al. (2002) report that brown trout can expel mature *M. cerebralis* myxospores while alive. In all cases, spores released into the environment can be ingested by the oligochaete host *T. tubifex*, invade its mucosa epithelia and develop interepithe-lially to the triactinomyxon stage in nearly 90 d.

Parasites such as *Myxobolus cerebralis*, which have unique life cycles, shuttling between vertebrate and in-vertebrate hosts, encounter harsh and varied environ-

ments, both in host-tissue and in free-spore stages. The survival strategies of these parasites frequently involve the participation of glycoconjugates, which can be used to build protective structures and to facilitate host–parasite interactions. Yet given the wide variety of environmental conditions faced and host adaptation over time to ward off parasite attack, the proteins and glycoconjugates of the parasite must also adapt over time if the organism is to survive. Fish immune systems are mainly based on non-specific immune responses (Ingram 1980) and frequently involve lectin activity in microbial defence (Hosono et al. 1999, Muramoto et al. 1999, Honda et al. 2000). The immune system has to be able to effectively recognise parasite glycan epitopes, in order to provide some resistance to infection. To counter the host response, protozoans rely on antigen mimicry (Damian 1987, Inal 2004) and antigen variation (Borst et al. 1996, Rudenko et al. 1998), processes which involve surface glycoproteins such as glycoepitopes of N-glycosides (Burghaus et al. 1999, Yang et al. 1999) and O-glycosides (Dieckmann-Schuppert et al. 1993, Khan et al. 1997). In *Trypanosoma brucei* (Borst et al. 1996, Ferguson 1997, 1999) and *Giardia lamblia* (Gillin et al. 1990, Gillin & Reiner 1996, Nash 1992), a variant surface glycoprotein (VSG) is expressed sequentially to evade the host's immune reaction.

A similar invasion strategy is likely with *Myxobolus cerebralis.* A number of studies on protozoan and metazoan parasites implicate glycans and lectins in the complex system of host recognition and pathogenesis (Jacobson & Doyle 1996, Loukas & Maizels 2000, Buchmann & Lindenstrøm 2002). Histochemical studies pointing to carbohydrate motifs on myxozoan parasites have been reported by Marin de Mateo et al. (1996), Morris & Adams (2004) and Knaus et al. (2005). These and further studies (e.g. Petri et al. 2002) discuss wider participation of glycans in adhesion and invasion processes, as well as in parasite tissue tropism (Bonilha et al. 1995, Ortega-Barria & Boothroyd 1999). Lectin blotting is a common analytical method to investigate and separate these glycoproteins, which have been extracted from parasites (Muñoz et al. 2000, Cohen et al. 2002, Gruden-Movsesijan et al. 2002, d'Avila-Levy et al. 2004). This method also can detect glycan epitopes in a highly sensitive and specific manner.

The current investigation was aimed at characterising and isolating glycoproteins from the myxospore stage of *Myxobolus cerebralis*, isolated from infected rainbow trout. Our methodology was generally based on analysis with biotin-labelled plant lectins and lectin-affinity chromatography, chosen according to their sugar specificity to identify oligosaccharide moieties. Isolated glycoproteins were analysed using peptide mass fingerprints acquired by matrix-assisted laser desorption/ionisation-mass spectrometry (MALDI/MS).

MATERIALS AND METHODS

Myxobolus cerebralis **myxospores.** Laboratory-infected rainbow trout *Oncorhynchus mykiss* were overdosed with the anaesthetic MS 222 (Sigma-Aldrich Chemical) and dissected. The skeleton of the fish was separated and homogenised in a commercial mixer for about 10 min. Suspended spores were then separated and concentrated by means of a plankton centrifuge according to the method described by O'Grodnick (1975), then further purified using a Percoll gradient. In a 15 ml centrifuge tube, Percoll at various concentrations (0–25–50–75–100%) was carefully layered; then, 2.5 ml of the spore sample was added. The tube was centrifuged in a swinging bucket rotor at $400 \times g$ for 10 min. Spores were concentrated into a white band, which was collected from the interface, diluted in 14 ml distilled water and washed 3 times to eliminate the Percoll solution and to further concentrate the spores.

Glycoprotein sample. A pellet of *Myxobolus cerebralis* spores ($\sim 1.5 \times 10^6$) was suspended in sample buffer (100 mM Tris-HCl, pH 6.8, 200 mM 2-mercaptoethanol, 4.0% v/v SDS, 0.2% w/v bromophenol blue, 20% v/v glycerol; Laemmli 1970), and the spores were lysed using a water bath sonicator (Transsonic 310; Elma) 10 times for 2 min; after each 2 min sonication, the sample was incubated on ice for 1 min. The suspension was then boiled at 95°C for 5 min and centrifuged at $14\,000 \times g$ for 3 min. The supernatant was collected carefully and washed 3 times with distilled water on a Microcon Centrifugal Filter Device YM3 (3000 kD; Millipore) to remove buffer and to concentrate the glycoproteins. The samples were aliquoted into microfuge tubes and stored at –80°C.

Discontinuous and reducing SDS-PAGE. An equal volume of 2× SDS loading buffer (see Laemmli 1970) was added to a glycoprotein sample, then boiled at 95°C for 5 min. After centrifugation at $14\,000 \times g$ for 3 min, soluble proteins were run on a 12% polyacrylamide resolving gel and a 5% stacking gel (Sambrook & Russell 2001). Running gels (10 ml) were polymerised by the addition of 100 µl of 10% (w/v) ammonium peroxodisulphate (APS) and 8 µl of N,N,N',N'-tetramethylethylenediamine (TEMED). Stacking gels (5 ml) were polymerised by the addition of 40 µl APS and 4 µl TEMED. All gels were run using a Mini-PROTEAN II electrophoresis cell (Bio-Rad Laboratories) at 130 V constant voltage and running buffer (25 mM Tris, 250 mM glycine, 0.1% w/v SDS, pH 8.3).

Electrophoretic transfer. After electrophoresis, proteins were transferred to a 0.45 µm PROTRAN nitrocellulose transfer membrane (Schleicher & Schuell BioScience) using a constant voltage of 100 V for 1 h, with transfer buffer (48 mM Tris, 38 mM glycine, 0.037%

v/v SDS, 20% v/v methanol; Sambrook et al. 1989) in a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad Laboratories).

Lectin blotting. Glycoprotein detection was performed with biotin-labelled plant lectins (listed in Table 1) and applied at the following concentrations: PHA-E 2.0 µg ml^{-1}, GSA I 2.0 µg ml^{-1}, GSA II 2.0 µg ml^{-1}, SJA 2.5 µg ml^{-1} (Vector Laboratories). Immediately after electro-transfer, unoccupied protein-binding sites on the membrane were blocked by incubation with BSA (bovine serum albumin, Sigma Chemical) blocking solution (3% w/v BSA, 150 mM NaCl, 10 mM Tris-HCl, 0.05% v/v Tween20, pH 7.5) overnight at 4°C with agitation. Membranes were then incubated with the lectins (2 µg ml^{-1} in blocking solution containing Ca^{2+}/Mn^{2+} [3% w/v BSA, 150 mM NaCl, 10 mM Tris-HCl, 0.05% v/v Tween20, 10 mM CaCl$_2$, 10 mM MnCl$_2$, pH 7.5]) for 2 h at room temperature with gentle agitation. The membranes were washed 3 times each for 10 min with TTBS (150 mM NaCl, 10 mM Tris-HCl, 0.05% v/v Tween20, pH 7.5), then incubated with 0.2 µg ml^{-1} streptavidine conjugated with horseradish peroxidase (Sigma Chemical) in blocking solution, for 1 h at room temperature with agitation. Membranes were again washed 3 times each for 10 min with TTBS. Bound lectins were visualised by ECL detection (Amersham Pharmacia Biotech) and documented on a CL-XPosure film (Perbio Science).

Lectin-affinity chromatography. Extracted glycoprotein samples were dissolved in 500 µl TBS. Then, 100 µl of lectin-conjugated agarose-matrix suspension was washed 4 times with TBS washing solution (TBS containing Ca^{2+}/Mn^{2+} [150 mM NaCl, 10 mM Tris-HCl, 1 mM CaCl$_2$, 1 mM MnCl$_2$, pH 7.5]). The 2 solutions were mixed in a 2 ml microfuge tube, then incubated overnight at 4°C with constant rotation. The tube was gently centrifuged 4 times at 700 × g for 3 min, between each centrifugation it was rinsed with the washing solution for 15 min. Elution was performed with TBS containing corresponding competitive ligands (PHA-E: 100 mM acetic acid; SJA: 200 mM N-acetyl-D-galactosamine; GSA I: 200 mM β-D-galactose, 200 mM N-acetyl-D-galactosamine; GSA II: 200 mM N-acetyl-D-glucosamine) overnight at 4°C with agitation. The eluate was collected after centrifugation (700 × g for 3 min) and washed 4 times with distilled water in a Microcon Centrifugal Filter Device YM3 (3000 kDa; Millipore). An SDS-PAGE was performed as described above. Lectin-affinity chroma-tography on extracted *Oncorhynchus mykiss* cerebral proteins served as a control glycoprotein source.

Deglycosylation. Specificity of lectin binding was cross-checked by comparing the performance of deglycosylation of *Myxobolus cerebralis* protein samples using a N-Glycosidase F Deglycosylation Kit (Roche Diagnostics) to hydrolyse N-glycosides from asparagine-coupled protein. Deglycosylation was performed in a 50 ml centrifuge tube (Carl Roth) after transfer to a nitrocellulose membrane and blocking with 3% BSA as described previously; 4 ml deglycosylation solution was prepared following Haselbeck & Hösel (1988) (4.0 U ml^{-1} N-glycosidase F, 50 mM sodium phosphate, 50 mM EDTA [ethylenediaminetetraacetic acid], 10 mM sodium azide, 3.0% w/v BSA, 0.5% v/v Triton X, 0.05% v/v Tween20, pH 7.0). Membranes were incubated for 12 h at 37°C with agitation, then washed 3 times each for 10 min in TTBS. Then, we processed according to the lectin blot protocol described above.

Protein staining. SDS-PAGE bands arising from affinity-purified glycoproteins and myxospore total protein were visualised with a Proteosilver staining kit (Sigma-Aldrich Chemie). Total *Myxobolus cerebralis* protein was detected by Coomassie Brilliant Blue R-250.

Mass-spectrometry. Glycoproteins, isolated using lectin-affinity chromatography (described above), were quantified using a peptide mass fingerprint. Briefly, protein bands were excised from the gel, digested with trypsin and subjected to mass spectrometry (TOPLAB) using a Voyager-DE STR (Applied Biosystems). Peptide mass fingerprint data were used to search for corresponding proteins in NCBI databases (www.ncbi.nlm.nih.gov/Entrez), including Swissprot protein and non-redundant databases

Table 1. Plant lectins used in this study

Lectin	Abbreviation	Monosaccharide specificity	Potent oligosaccharide
Griffonia simplicifolia Agglutinin I	GSA I	GalNAc	GalNAcα3Gal, GalNAcα3GalNAcβ3Galα4Galβ4Glc
Griffonia simplicifolia Agglutinin II	GSA II	GlcNAc	GlcNAcβ4GlcNAc
Phaseolus vulgaris erythroagglutinin	PHA-E	No known specificity	Bisected complex-type N-glycans, Galβ4GlcNAcβ2 Manα6(GlcNAcβ2Manα3)(GlcNAcβ4)Manβ4GlcNAc
Sophora japonica agglutinin	SJA	GalNAc	GalNAcβ6Gal

Immunoblotting. Glycoproteins of *Myxobolus cerebralis* and those eluted with lectin-affinity chromatography were used to perform immunoblotting analyses with anti-triactinomyxon polyclonal antibodies produced in rabbit (anti-TAMpcAb). After separation on SDS-PAGE and transforming to a nitrocellulose membrane (described above), the remaining binding sites of the membrane were blocked with 5% w/v skim milk in TTBS for 2 h at room temperature with agitation. The membrane was then incubated with anti-TAMpc antibodies 1:1000 (primary antibody) in blocking buffer for 2 h (room temperature, agitation). After washing 3 times with TTBS, 15 min each, the membrane was incubated with anti-rabbit antibodies conjugated with horseradish peroxidase (secondary antibody, Sigma-Aldrich Chemie) diluted in blocking buffer for 2 h (room temperature, agitation). After 3 additional 15 min washings in TTBS, bound antibodies were visualised by the ECL system and documented on CL-XPosure film. Proteins extracted from TAM spores and *Oncorhynchus mykiss* cartilage were used as controls. Rabbit control antiserum (1:500 diluted) was used on further blots to detect binding of antibodies not specific for TAM antigens.

RESULTS

Lectin blot analyses

The glycoprotein composition of the myxospore total protein of *Myxobolus cerebralis* was examined by binding with corresponding biotin-labelled plant lectins (Table 1), giving rise to banding patterns shown in Fig. 1, showing control non-infected *Oncorhynchus mykiss* glycoproteins (Fig. 1, Lane 1a) next to myxospores (Fig. 1, Lane 1b). Specificity controls were included to quantify any binding effects of streptavidin-peroxidase, endogen avidin and peroxidase

activities. The glycoprotein bands at about 70 kDa were due to endogenous streptavidin-binding activity. Blot affinity studies with GSA I (Lanes 1a, 1b) detected GalNAcα3-terminal epitopes on myxospore proteins with molecular weights between 10 and 210 kDa. In contrast, GSA II bound to glycans containing GlcNAc moieties with molecular weights <18 kDa (Lane 2b), with weaker bands corresponding to proteins of 23, 27 and 36 kDa. SJA bound to epitopes carrying terminal GalNAcβ6: 5 to 18 kDa glycoproteins (Lane 3b), with additional binding of 23-, 27-, 33-, 40-, 48- and 60-kDa proteins. PHA-E bound a broad variety of higher weight myxospore glycoproteins, 12 to 210 kDa (Lane 4b), with distinct bands at 19, 23, 48 and 100 kDa.

Deglycosylation

N-glycosidase F was used to verify the specificity of lectin binding. In a modification of the routine protocol, deglycosylation was performed after transfer to a nitrocellulose membrane. The lectin-affinity profile of isolated *Myxobolus cerebralis* glycoproteins after deglycosylation is shown in Fig. 1 (Lanes 1c, 2c, 3c and 4c). Deglycosylation appeared to prevent lectin binding in most cases. With GSA II (Lane 2c) only a single 4 kDa band remained. Weak bands were also evident with SJA (Lane 3c) at 4, 19, 21 and 33 kDa.

Lectin-affinity chromatography

Glycoproteins isolated by lectin-affinity chromatography were separated on SDS-PAGE and visualised with silver staining (Fig. 2). The procedure was performed in parallel with control-protein extracts from *Oncorhynchus mykiss*, to prevent any misinterpretation of resultant bands. Affinity chromatography with the GSA II matrix (Lane 2b) resulted in the isolation of
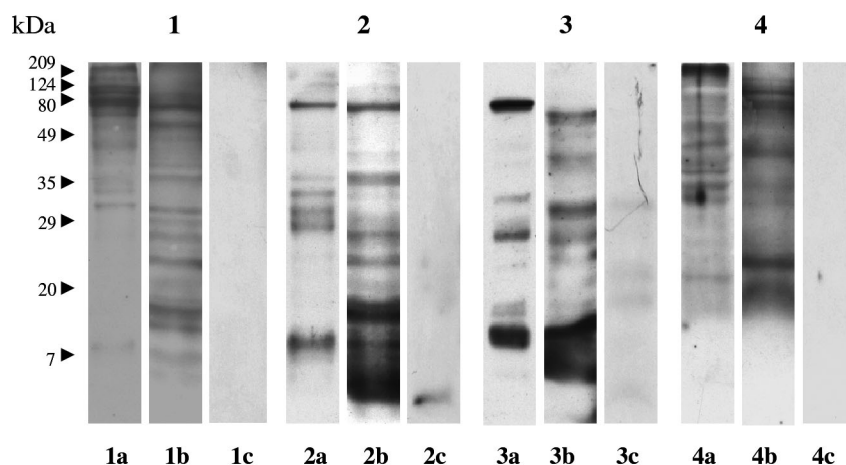

Fig. 1. Binding pattern profiles of glycoproteins in discontinuous 12% SDS-PAGE on 3 protein sources: (Lanes a) head cartilage of *Oncorhynchus mykiss*, (Lanes b) *Myxobolus cerebralis* myxospores and (Lanes c) deglycosylated *M. cerebralis* myxospores; using 4 different lectins, GSA I (1), GSA II (2), SJA (3) and PHA-E (4). Arrowheads indicate molecular weights of standard proteins

a single myxospore glycoprotein at 29 kDa and showed similar results to trout cartilage proteins (Lane 2a), with bands at 28 and 31 kDa. With SJA (Lane 3b)-conjugated agarose matrix, a 33 kDa glycoprotein was purified and named Mcgp33. Host cartilage did not generate any comparable bands. No myxospore glycoproteins could be isolated utilising GSA-I (Lane 1b) or PHA-E-conjugated agarose matrix (Lane 4b).

Mass-spectrometry

Matrix-assisted laser desorption/ionisation−mass spectrometry (MALDI/MS) was performed on 3 proteins excised from the affinity gels: 29 kDa (Fig. 2, Lane 2a), 29 kDa (Lane 2b) and Mcgp33 at 33 kDa (Lane 3b). The MALDI/MS peptide mass results from the Lane 2a and 2b bands showed significant correlation. The results from Mcgp33 are shown in Fig. 3. Peptides with molecular weights of 1111.5254, 1433.7130, 2163.0570, 2273.1765 and 2550.2766 (m/z) are known contaminant autolysis products of trypsin. Specific prominent peaks remained at 615.3208, 713.3554, 788.3769, 999.5171 and 1138.5799 (m/z). A database search using these mass data did not indicate any known peptides.
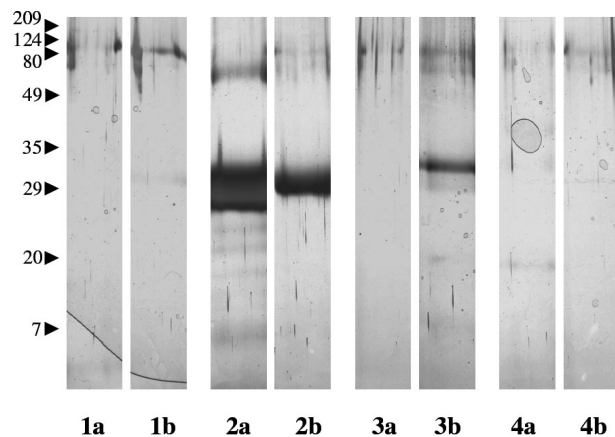

Fig. 2. Silver-stained reducing discontinuous 12% SDS-PAGE of glycoprotein samples prepared by lectin-affinity chromatography, with proteins from (Lanes a) *Oncorhynchus mykiss* and (Lanes b) *Myxobolus cerebralis* myxospores. Lanes 1a, 1b: GSA I showed no significant affinity with either *O. mykiss* (1a) or *M. cerebralis* (1b). Lanes 2a, 2b: GSA II showed distinct *O. mykiss* glycoprotein bands at 28 and 31 kDa (2a) and a *M. cerebralis* glycan (29 kDa) highly decorated with GlcNAcβ4GlcNAc residues (2b). Lanes 3a, 3b: SJA. *O. mykiss* showed no affinity to SJA (3a), while *M. cerebralis* (3b) contains a glycoprotein highly decorated with GalNAcβ6Gal epitopes (33 kDa). Lanes 4a, 4b: PHA-E showed no significant affinity with either *O. mykiss* (4a) or *M. cerebralis* (4b). Arrowheads at left indicate molecular weights of standard proteins

Electrophoresis and immunoblotting

Myxospore proteins separated by SDS-gel electrophoresis gave bands covering a broad molecular weight range between 7 and 200 kDa (Fig. 4, Lane 1). Lanes 2 and 3 show the results of blotting anti-triactinomyxon polyclonal antibodies (anti-TAMpcAb, produced in rabbit) against glycoprotein extracts from both spore stages of *Myxobolus cerebralis*. Antibody reactions on myxospore proteins (Lane 2) had molecular masses of 29 to 210 kDa, with dominant bands at 30, 40, 70, 100 and 125 kDa, and a less-intense band at 7 kDa. The antigen giving rise to the 70 kDa band was found to possess endogen streptavidin-binding activity (described above). Anti-TAMpcAb had broad reactivity to proteins of triactinomyxon spores. Lectin-affinity chromatography results are shown in Fig. 2 (Lanes 2b and 3b). Neither GSA II (Lane 4) nor SJA (Lane 5) showed binding of anti-TAMpcAb.

DISCUSSION

Although a fair amount is known about morphological characteristics of the development and lifecycle of *Myxobolus cerebralis*, much remains to be elucidated regarding the biochemistry of attachment processes of parasite spores to host cells. Carbohydrate motifs in *M. cerebralis* spores, in particular, interact with binding sites, both on rainbow trout and oligochaetes, and provoke immune responses: processes of glycan recognition that are often mediated by lectins. Buchmann (2001) notes that the mucus of rainbow trout has lectin activity for mannose, galactose and lactose. Knaus et al. (2005) use lectin histochemistry as an indicator of carbohydrate motifs in *M. cerebralis* spores. In the present study, we identified glycoproteins in *M. cerebralis* myxospores through detection with plant lectins. These glycoproteins, putatively responsible for inducing a host immune response, were tested against antigens derived from glycoproteins of *M. cerebralis* actinospores (triactinomyxons).

Each of the 4 plant lectins used led to detection of specific glyco-motifs in the myxospore protein extracts. Several bands were detected by >1 lectin, which indicates the presence of multiple oligosaccharide structures. In particular, 23 kDa glycoproteins were reactive with all lectins. Reactivity to *Phaseolus vulgaris* erythroagglutinin (PHA-E) indicates the presence of trisaccharide units [β-D-Gal(1-3,4)-β-GlcNAc(1-2)-D-Man] in so-called bisected complex-type N-glycans (Goldstein & Hayes 1978); these branched N-acetyl-D-glucosamine modifications were present in multiple myxospore glycoproteins. *Griffonia simplicifolia* agglutinin II (GSA II) binds with residues containing N-
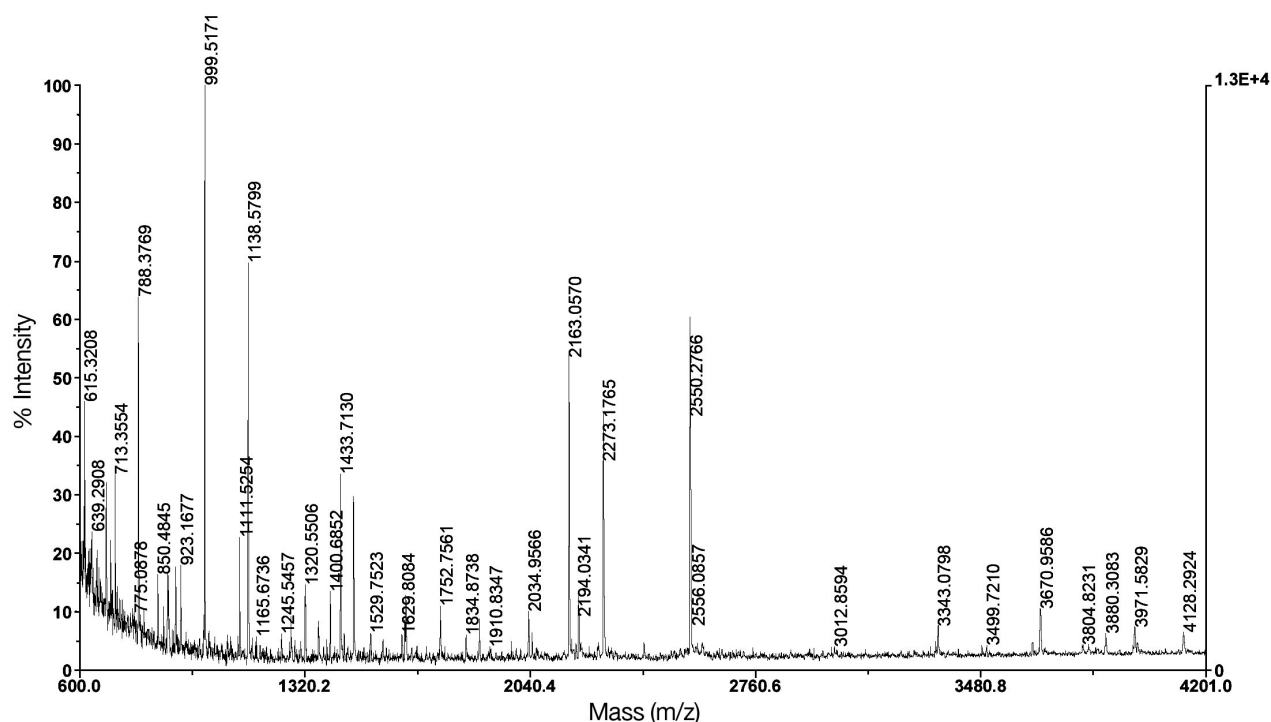
Fig. 3. Peptide-mass fingerprint showing Mcgp33 band resulting from protein separation after SJA-affinity chromatography with *Myxobolus cerebralis* myxospores
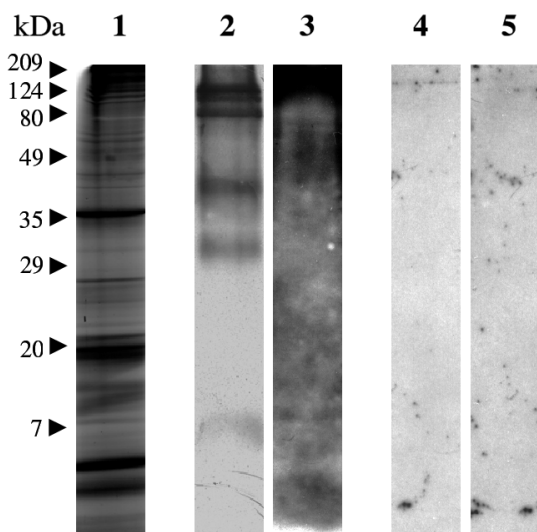

Fig. 4. Lane 1: Coomassie brilliant blue stain of total protein extracted from developmental stages of *Myxobolus cerebralis* in *Oncorhynchus mykiss* following separation by 12% SDS-PAGE. Lanes 2, 3: antigen detection profiles of *M. cerebralis* myxospores (2) and actinosporeans (3) against anti-TAMpcAb (rabbit) myxospore antigens reacted to produce banding at 9 and 30 to 225 kDa. Lanes 4, 5: antigen detection profiles of glycoproteins isolated from *M. cerebralis* by lectin-affinity chromatography against SJA (4) and GSA II (5). Arrowheads at left indicate molecular weights of standard proteins

acetyl-D-glucosamine and GlcNAcβ4GlcNAc, which were located at sites on polar capsules and amoebic germs, respectively (Knaus et al. 2005). Almost all of the glycoconjugates we detected were N-glycosylated. We isolated a SJA-binding glycoprotein, Mcgp33, which subsequent studies revealed was not detectable by anti-triactinomyxon antibodies. Anti-TAM-reactive antigens in myxospores generally have higher molecular masses.

Plant lectins have proven to be extraordinarily useful tools for investigating carbohydrate structure, because of their affinity for specific terminal and internal sugars, carbohydrate linkages and oligomers, in complex glycans. Lectin blotting has already been used both on purified membrane proteins (Jaffe & McMahon-Pratt 1988) and crude membrane extracts of *Leishmania* promastigtes (Grogl et al. 1987, Rossell et al. 1990). In our case, plant lectins were selected based on the histochemical results of Knaus et al. (2005). Based on morphological observations, the lectins indicated the existence of complex N-glycans located on shell valves, amoebic germs and polar capsules of myxospores in fish cartilage. Epitopes containing both GalNAcα3- (GSA I) and GalNAcβ6-residues (SJA) were detected on polar capsules and amoebic germs, but not on shell valves.

We also developed a deglycosylation method that allows hydrolysis to be done after the proteins have

been transferred to a nitrocellulose membrane. This involved modifying buffers, as noted in the 'Materials and methods' section. A similar approach using polyvinylidene fluoride (PVDF) membranes has been developed by Weitzhandler et al. (1993) for determination of the complete monosaccharide composition of selected proteins. Our modifications to this procedure allow simultaneous comparison of molecular weights of native and deglycosylated samples; otherwise, bands may shift because of mass loss before running in the gel.

Differences in composition between the carbohydrate moieties of the host and invading pathogen are also fundamental to the function of an innate immune system — the discrimination of self from non-self (Turner 1996). Immunity defence strategies of fish, principally non-specific, rely on successful detection of foreign molecules, especially proteins, the antigenicity of which may be based on both their underlying amino acid structure, as well as post-translational modifications such as glycosylation (Feizi & Childs 1987). Specific immune responses in a fish host may be triggered by glycosylated parasite proteins, released after spore lysis or other contact.

The first barrier a fish presents to an invading pathogen is the mucous layer of the epidermis. Analyses of mucus from several fish species, including eel, conger and rainbow trout, have revealed various lectin activities, suggesting a defensive role is played by some of the component molecules of the mucus (Hosono et al. 1999, Muramoto et al. 1999, Honda et al. 2000, Buchmann 2001), although the specific functions of these lectin activities have not been fully elucidated. Epitopes from *Myxobolus cerebralis* myxospores contain so-called bisected N-acetyl-D-glucosamine. This is a core modification of complex N-glycans (Goldstein & Hayes 1978) known to be catalysed by N-acetyl-D-glucosamintransferase III (GnT-III), a process which may involve either the parasite's own enzyme activities or the host's own enzyme portfolio. Many studies indicate utilisation of host enzymes by parasites, in order to evade the host's immune response through antigen mimicry (Damian 1987, Inal 2004). With this in mind, we questioned whether *M. cerebralis* has developed a similar strategy of host mimicry. We have shown that Mcgp33 is expressed in *M. cerebralis* myxospores in the fish host, but not in the triactinomyxon stage, which serves as evidence in favour of Markiw's (1989) postulates of adaptive expression of *M. cerebralis* antigens. Yet other antigens, detected by Hamilton & Canning (1988) and Markiw (1989), were present in both spore stages from fish and oligochaete hosts. We can confirm previous histochemical findings that there is variation of presented carbohydrate moieties in the 2 spore stages (Knaus et al. 2005).

After release from the fish host, *Myxobolus cerebralis* myxospores are ingested by the oligochaete host, *Tubifex tubifex*, in which they then invade the gut epithelia (El-Matbouli & Hoffmann 1998). An invading strategy specific for the target tissue of the host may be based on the distinction between lectins and glycans (Jacobson & Doyle 1996, Loukas & Maizels 2000, Buchmann & Lindenstrøm 2002). In a similar manner to the fish host, lectin molecules are suggested to play an important role in the immune response of invertebrates (Bilej et al. 2000), although no data are available on lectin activity in the gut epithelial cells of *T. tubifex*. The role of the glycan–lectin system in *M. cerebralis*' invasion of *T. tubifex* is undoubtedly worth additional investigation, as it may shed new light on the divergent susceptibilities of different strains of *T. tubifex* to the parasite (Beauchamp et al. 2002).

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