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Carbohydrate localization on *Gyrodactylus salaris* and *G. derjavini* and corresponding carbohydrate binding capacity of their hosts *Salmo salar* and *S. trutta*

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Abstract

The congeners *Gyrodactylus salaris* and *G. derjavini* are specific ectoparasites of Atlantic salmon *Salmo salar* and brown trout *S. trutta*, respectively. To elucidate the involvement of lectin–carbohydrate interactions in this host specificity, carbohydrates on the tegument of the two species and the corresponding lectin activity of their hosts have been studied. Carbohydrate composition on the tegument differed significantly between the two gyrodactylids. Three of four commercially available peroxidase-labelled lectins with primary affinity towards D-mannoside, D-GalNAc and L-fucose bound more strongly to *G. derjavini* than to *G. salaris*. Lectins with an affinity towards D-mannoside and D-GalNAc bound significantly stronger to the cephalic lobes on *G. derjavini* compared to the tegument and sheaths of the hamuli. One brown trout strain and three different salmon strains were tested for lectin activity in skin and plasma. Two Baltic salmon strains and one strain from the Atlantic region were included. Brown trout differed significantly from the salmon strains when skin samples were tested for D-GalNAc activity. Lectins binding to other carbohydrates showed trends for similar host differences. The implications of carbohydrate–lectin interactions for host specificity in gyrodactylids are discussed.

Introduction

Host specificity of gyrodactylids on teleosts is highly developed. This is particularly well-characterized and evident in salmonid hosts. Thus, *Gyrodactylus salaris* Malmberg, 1957 and *G. derjavini* Mikailov, 1975 have the Atlantic salmon *Salmo salar* and the brown trout *Salmo trutta*, respectively, as their primary hosts (Mo, 1991, 1993; Buchmann & Uldal, 1997; Bakke *et al.*, 1999). In addition, several studies have shown that the susceptibility to *G. salaris* differs between different strains of Atlantic and Baltic stocks of *S. salar*, Baltic salmon being less susceptible than Atlantic salmon (Bakke *et al.*, 1990, 2002; Cable *et al.*, 2000; Dalgaard *et al.*, 2003). However, the host–parasite interactions that lead to different preferences of *Gyrodactylus* species for fish hosts are inadequately described. Lectins and their abilities to recognize specific carbohydrate conformation have been suggested to explain host–parasite specificity (Buchmann, 2001). Thus the presence of lectins in fish skin is of common occurrence which suggests that protein–carbohydrate interactions could be involved in host recognition. Recent studies have shown that galectins are found in skin mucus from the conger eel *Conger myriaster* (Muramoto *et al.*, 1999) and collectins with different specificity to carbohydrates have been found in the spleen of *Cyprinus carpio* (Vitved *et al.*, 2000). In addition, host complement proteins were previously found to bind *G. derjavini* and *G. salaris* from rainbow trout, brown trout and salmon (Buchmann, 1998; Harris *et al.*, 1998). The present

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work aims to elucidate whether carbohydrate–lectin interactions differ among different host stocks and parasites, and whether this could explain why *G. derjavini* and *G. salaris* differ so clearly in their host preferences. Thus, the binding of lectins to specific carbohydrate epitopes on the tegument of these two congeners and corresponding lectin activities in skin extracts and plasma from their respective hosts are described.

Materials and methods

Parasites

Gyrodactylus salaris originating from the river Lærdalselva in Norway was maintained as a laboratory stock on susceptible Scottish salmon (Dalgaard *et al.*, 2003). *Gyrodactylus derjavini* from a trout farm (Paelebro) in Jutland was maintained as a laboratory stock on susceptible rainbow trout (Buchmann & Uldal, 1997). Both salmon and trout were killed with MS222 (A-5040, Sigma, USA) and fixed at 12°C in neutral formalin for 24 h whereupon parasites were isolated from the fish. Isolated parasites were incubated with fresh formalin and stored at 5°C until use.

Fish

Salmonid fry 1+ (body length of 10–15 cm) were sampled in May 2003. Brown trout originated from Esrum Fish Production (North Zealand, Denmark). Salmon from the Conon river in Scotland (Atlantic strain), from the Lule river in Sweden and Russian Neva river (Baltic strains) were imported to the Danish isolated fish rearing facilities where recirculated freshwater is used.

Plasma

Fish were anaesthetized with MS222 before heparinized blood samples were taken from all fish by caudal vein puncture. Following centrifugation (13,000 rpm for 10 min Biofuge 13, Heraeus SEPATECH) plasma was recovered and diluted 1:200 in PBS (phosphate-buffered saline pH 7.2). Diluted samples were stored at −18°C until use. Samples from Lule salmon were pooled from ten fish because of small yields of blood.

Skin extracts

Skin samples were taken immediately after blood sampling. Samples were excised from the left side of each fish below the dorsal fin. Excision included the lateral line and the size was approximately 2 cm^2 (weight 0.051–0.125 g). Samples consisted of epidermis and dermis, but no muscle tissue. Skin samples were weighed and cut into small pieces and diluted 1:10 (1 g = 1 ml) with phosphate buffered saline (PBS), sonicated for 2 min with 20 pulses of 5 sec at 130W (Sonicator® ultrasonic proccesor XL 2020, Heat System, USA). Following centrifugation (13,000 rpm for 10 min, Biofuge 13, Heraeus SEPATECH) the supernatant was recovered and diluted to 1:400 with PBS. Diluted samples were stored until use at −18°C.

Lectin-binding to parasites

Formalin-fixed specimens of *G. salaris* and *G. derjavini* were twice rinsed in distilled water for 15 min and incubated 1 h in 2 ml wells at room temperature (in a shaded laboratory) in blocking buffer (PBS with 2% bovine serum albumin at pH 6.6). Subsequently they were incubated 1 h at room temperature with peroxidase labelled lectins (10 $\mu g\,ml^{-1}$) in blocking buffer (table 1). Seven to 29 worms were used in each group for each lectin and the experiments were repeated twice. Lectin from *Ricinus communis* was only used in the lectin inhibition assay. Following incubation with lectins, parasites were rinsed three times for 10 min each time with washing buffer (PBS with 0.05% TWEEN 20). Lectin-binding was visualized by adding enzyme substrate (diamino-benzidine-tetrahydrocloride D5905, Sigma, USA) and 12 μl 30% H_2O_2 dissolved in 15 ml PBS. The reaction was stopped after 5 min by rinsing with washing buffer and 5 min with distilled water. Worms incubated without peroxidase conjugated lectins, with specificities stated by the manufacturer, served as negative controls. However, competitive carbohydrate inhibition (to confirm specificities) was not conducted. Parasites were mounted on

Table 1. Peroxidase labelled lectins used both in the lectin inhibition assays and studies on lectin-binding to parasites. Major carbohydrate specificities of lectins and carbohydrates coated on Glycowell plates according to the manufacturer are shown. Lectins from *Ricinus communis* were not used in the lectin-binding to parasites assay.

Lectin, peroxidase labelled	Sigma no.	Primary specificity	GlycoWell™ no.	Carhohydrate on GlycoWell™
Canavalia ensiformis (Con A)	L6375	D-mannosyl and D-glucosyl residues	SW-01-009	D-mannoside
Triticum vulgaris	L3892	N-acetyl-D-glucosaminyl residues and N-acetyl-D-glucosamine oligomers	SW-01-002	N-acetyl-D-galactosaminyl (2-acetamido-2-deoxy-D-galactopyranoside)
Maclura pormifera	L4401	Terminal D-galactosyl and N-acetyl-D-galactosaminyl residues	SW-01-007	D-galactose
Ulex europaeus	L8146	L-fucose (6-deoxygalactose)	SW-01-005	L-fucose (6-deoxygalactose)
Ricinus communis	L2758	Terminal D-galactosyl residues	SW-02-001	Lactoside (O-D-galactopyranosyl-(1-4) -D-glycopyranose)

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microscope slides in glycerine gelatine and studied under a compound microscope (Leica DMLB, Leica Microsysten, Germany) at 200 to 400 × magnification. The intensity of the coloration was registered on an ordinal scale from 0 to 5, where 0 was no coloration and 5 strong brownish. Cephalic lobes, the tegument, upper hamuli sheaths and lower hamuli sheaths were the areas which were observed.

Lectin inhibition assay (fish skin and plasma)

The binding of peroxidase labelled lectins to immobilized carbohydrates and subsequent inhibition of such a binding event were used for the evaluation of lectin activity in skin and plasma samples. Microtitre GlycoWell™ plates (Lundonia Biotech™, Lund Sweden) with covalently linked carbohydrates were used together with peroxidase labelled lectins (table 1). The system expresses lectin activity in a sample by quantifying its competitive inhibition of the binding of peroxidase labelled lectin to a carbohydrate-coated well. A strong colour development signals maximum binding of the peroxidase labelled lectins and thus no inhibition by the sample. A low coloration indicates that the sample added has inhibited the binding of the peroxidase labelled lectins to the well (Buchmann, 2001). Reactions were run at room temperature in a shaded laboratory. A volume of 50 μl lectin solution (10 μg lectin per ml in PBS with 0.05% TWEEN 20 filtered through a 0.22 μm filter) (PBS/TWEEN) were incubated with 50 μl samples of skin or plasma at pH 7.2. The level of pH was altered to pH 6.6 when concanavalin A (Con A) was used because Con A will aggregate at a pH above pH 7.0. After 45 min incubation each well was washed three times with 150 μl Cova buffer (pH 7.0 (Con A pH 6.6):116.9 g NaCl, 10.0 g $MgSO_4.7H_2O$, 0.5 ml TWEEN 20, 1 litre PBS). The last wash was left for 15 min before it was removed, then each well was washed once with citrate/phosphate buffer (pH 5.0: 7.30 g citric acid, 11.86 g $Na_2HPO_4.2H_2O$, 1 litre H_2O). Substrate solution 100 μl (6.0 mg o-phenylenediamine (P8806, Sigma, USA), 10.0 ml citrate/phosphate buffer, 5 μl H_2O_2, freshly prepared) was added and incubated for 15 min. The reaction was stopped with 100 μl 3.0% H_2SO_4 and absorbance was read at 492 nm in an enzyme linked immunosorbent assay (ELISA) reader (Multiscan RC, type 351, Labsystems, Finland). Maximum binding was achieved by adding 50 μl of lectin and 50 μl of PBS/TWEEN per well in the first step. The background was measured by adding 100 μl PBS/TWEEN per well and without peroxidase-labelled lectin.

Protein determination (fish skin and plasma)

The protein concentration in each sample of skin and plasma was measured in duplicate by the bicinchoninic acid (BCA) method. Samples of 10 μl were incubated with 200 μl BCA solution (1:50 copper sulphate: bicinchoninic acid solution 23227, Pierce Biotechnology, USA) in 96-well microtitre plates. Prediluted bovine serum albumin (BSA) solutions (Pierce Biotechnology, no. 23209) were used according to the manufacturer's recommendation as standards. The background was measured using wells with only 210 μl BCA-reagent. Plates were incubated for 45 min at 37°C and subsequently read at 560 nm in an enzyme linked immunosorbent assay (ELISA) reader.

Statistical analysis

The Fisher exact test was used to evaluate results from the lectin-binding assay by comparing two groups. In the lectin-inhibition assay the percent inhibition calculated from the ELISA-reading was corrected for background and divided by the protein concentration achieved from the protein determination, to give an outcome to be statistically tested as the percent inhibition per microgram protein in each sample (% inhibition/μg protein). Analyses of variance were used to evaluate the outcome from the lectin-inhibition assay. The procedure PROC MIXED in the statistical program SAS® System (version 8.2) was used to evaluate the results from the lectin inhibition assay with an analysis of variance. All models were tested with fish length and the interaction of fish length with the different species. For pair-wise comparisons t-tests were used. Residuals were tested for normality using the Shapiro-Wilks test for normality. Equal variance was observed in a residual plot. All tests had a probability level of 0.05 and were performed in the SAS® System 8.2.

Results

Lectin-binding to parasites

Lectins (concanavalin A (Con A), *Triticum vulgaris* and *Ulex europaeus*) bound generally stronger to *G. derjavini* than to *G. salaris*. Con A- and *Triticum vulgaris*-lectins bound more strongly to the cephalic lobes of *G. derjavini* than the three other sites (table 2). *Maclura pormifera* lectin bound strongly to all sites, but small significant differences were observed in the binding to the hamuli sheaths. Thus, this lectin bound more to the upper hamuli sheaths on *G. derjavini* than any other site, and on *G. salaris* the lectin bound only weakly to the lower hamuli sheaths. Only a few negative control parasites (incubated without peroxidase conjugated lectins) were observed to be stained at level 1 on the ordinal scale (slight background staining). All other controls were at level 0. All areas except the lower hamuli sheaths on *G. derjavini* were significantly more coloured than corresponding areas on *G. salaris*. Values of $P < 0.001$ were obtained for the tegument, cephalic lobes and upper hamuli sheaths, and a P value of 0.0806 for lower hamuli sheaths. On both species, the *Triticum vulgaris*-lectin bound more strongly to the cephalic lobes than to other sites.

Lectin inhibition assay (fish skin and plasma)

All analyses were tested with fish length and interactions of fish length with the different species/strains in the model, and if no effects from these variables were found to be significant, they were therefore eliminated. All tests were normally distributed and the homogeneity of variance was evaluated to be equal in each residual plot. The inhibition of lectin from *Triticum vulgaris* was statistically significant when skin samples were used

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Table 2. The binding strength of lectins to *Gyrodactylus derjavini* and *G. salaris*. The median (ordinal scale) from observations in each area on each parasite species is shown. The number of parasites studied for per area ranged from 7 to 29.

Lectins	Species	Cephalic lobes	Tegument	Upper hamuli sheaths	Lower hamuli sheaths
Con A	*G. derjavini*	3 *a	3 *b	1 *c	1 *c
	G. salaris	1 a	1 a	0 c	0 c
Triticum vulgaris	*G. derjavini*	4 *a	3 *b	3 *bc	2 *c
	G. salaris	3 a	2 b	2 b	2 b
Maclura pormifera	*G. derjavini*	4 b	4 b	4 a	4 b
	G. salaris	4 a	4 ab	4 a	3 b
Ulex europaeus	*G. derjavini*	1 *	1 *	1 *	1 *
	G. salaris	0	0	0	0

*Significantly different lectin binding between the two parasite species in a particular area. Different letters denotes significantly different binding strength between areas on each parasite species.

Table 3. Inhibition of lectin binding to immobilized carbydrates in GlycoWells by adding samples from skin and plasma of *Salmo salar* and *S. trutta*.

			S. salar		
Lectins	Sample type	*S. trutta* Esrum ($n = 5$)	Lule ($n = 5$)	Neva ($n = 5$)	Conon ($n = 4$)
Con A	Skin extract	5.5 ± 2.3	3.4 ± 1.1	4.4 ± 1.0	4.2 ± 0.8
	Plasma	22.5 ± 2.7	16.7**	25.9 ± 4.6	27.7 ± 5.3
Triticum vulgaris	Skin extract	3.1 ± 1.53*	−0.4 ± 2.8	−0.7 ± 1.6	−2.4 ± 3.8
	Plasma	−6.5 ± 18.3	7.6**	−7.9 ± 23.1	11.8 ± 8.6
Maclura pormifera	Skin extract	5.9 ± 2.2	4.1 ± 1.2	5.2 ± 1.5	5.2 ± 0.5
	Plasma	5.4 ± 13.9	−8.1**	14.2 ± 11.8	23.4 ± 6.4
Ulex europaeus	Skin extract	6.6 ± 2.4	4.1 ± 1.2	5.2 ± 1.4	5.4 ± 0.7
	Plasma	19.5 ± 4.2	15.9**	22.9 ± 6.5	27.1 ± 5.0
Ricinus communis	Skin extract	0.4 ± 2.3	4.03 ± 1.1	5.0 ± 1.4	5.3 ± 0.7
	Plasma	18.1 ± 4.9	15.6**	21.2 ± 7.3	26.2 ± 5.1

The percentage inhibition of lectin binding to wells is expressed as % inhibition/μg protein (mean ± SD). *Significant difference between samples. **Samples are pooled from ten fish (Lule plasma samples only). *n*, the number of fish in each sample for each species or race.

($P < 0.05$). Brown trout skin samples inhibited more strongly than skin samples from the salmon strains (table 3). No other differences were found, but a trend towards higher inhibition by brown trout skin samples was noted. No significant differences were found in the plasma studies which generally showed a higher lectin activity than skin samples. A non-significant trend to increased inhibition by Conon river salmon plasma was observed (table 3).

Discussion

Results of lectin-binding to *G. derjavini* and *G. salaris* clearly indicated the presence of different carbohydrate conformations on the surfaces of both species. Three of four lectins bound significantly stronger to the surface of *G. derjavini* compared to *G. salaris*. This suggests that parasite–host interactions and specificity involve lectin–carbohydrate dynamics. Lectin binding to the two *Gyrodactylus* species also revealed different carbohydrate localization in different areas of *G. derjavini* and *G. salaris*. Con A and the *Triticum vulgaris*-lectin both bound significantly stronger to the cephalic lobes of *G. derjavini*. The binding of Con A to the cephalic lobes on *G. derjavini* has previously been shown by Buchmann (1998, 2001), who also showed that complement factor C3 binds to the same site and to the hamuli sheaths. Complement factor C3 could have bound to mannosyl residues or D-GalNAc, which are present on the cephalic lobes in both parasites. The upper hamuli sheaths on *G. derjavini* were significantly more coloured than other areas by *Maclura pormifera*-lectin, which have an affinity towards D-galactose. The lower hamuli sheaths on *G. salaris* were significantly less coloured than the other areas when this lectin was used. Results from lectin inhibition assays in fish skin and plasma samples showed that the plasma generally has a stronger inhibitory activity. Despite this, results from the skin (which is in direct contact with the gyrodactylids) should be considered more relevant for studies on interactions between these ectoparasites and the host. There was only one significant difference in host lectin activity when different hosts were compared. Thus, *Triticum vulgaris*-lectin binding was significantly more affected by trout skin which inhibited the lectin more than skin from the three strains of salmon. Although not statistically different, it is noteworthy that there was a general trend towards a stronger trout skin inhibition of all the lectins compared with the three strains of salmon. It is possible that the difference in carbohydrate

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localization on the two parasites and the corresponding different host lectin activity of their preferred hosts can, at least partly, explain host specificity of *G. derjavini* and *G. salaris* for brown trout and salmon, respectively.

Interactions between carbohydrates in parasites and lectins in fish can be speculated to be both positive and negative. Thus stimuli for appropriate feeding and reproduction in the parasite could be signalled by host lectins. However, negative and even lethal effects of host lectins are also possible because some lectins activate the host complement system which is known to affect gyrodactylids negatively (Buchmann, 1998; Harris *et al.*, 1998). Evidence has in fact been presented suggesting the involvement of immune parameters in salmonid susceptibility (Harris *et al.*, 2000). Further, galactose-specific lectins have been found in several teleost species and these can also initiate the lectin complement pathway. This has been suggested by Vitved *et al.* (2000) who found lectin homologues to mannose-binding lectin (MBL) in carp but with affinity towards galactose. This could then lead to activation of the lectin complement pathway and the killing of gyrodactylids. Only slight lectin differences between different strains of salmon were detected in this work but it is still possible that differences in the susceptibility of the different stocks from the Atlantic and the Baltic to *G. salaris* can be partly explained by differences in lectin composition. Thus, in the present work slight differences in plasma lectins were found between the Atlantic Conon river salmon with well described high susceptibility to *G. salaris* (Bakke & MacKenzie, 1993; Dalgaard *et al.*, 2003) and the Baltic Lule and Neva strains with lower susceptibility (Bakke *et al.*, 1990; Dalgaard *et al.*, 2003). Therefore, this issue should be further investigated. In this context other carbohydrate–lectin associations should also be included. One of several other carbohydrates is sialic acid which is known to have multiple roles in interactions between hosts and pathogens. The presence and absence of sialic acid on pathogens has previously been related to an increase in their ability to cause disease or infect host species (Varki, 1997; Yoshida *et al.*, 1997). Therefore it should be further elucidated if this and other carbohydrates and their corresponding lectins complement the results presented in the present work on gyrodactylid–teleost interactions.

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