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Surface Proteins and Glycoproteins of *Meloidogyne incognita*

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Abstract . The molecular weight of proteins and glycoproteins (gp) on the surface and in homogenates of second stage juvenile (J2) of *Meloidogyne incognita* have been investigated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Three main bands of easily extractable proteins characterise the surface of second stage *M. incognita*, but further eleven proteins of different molecular weight were apparently surface-related. Three of the main surface polypeptides were also observed in the whole nematode homogenate (205, 104, 44 kDa). The major glycoproteins were detected on the surface of J2 *M. incognita*. Comparison between J2 and female of *M. incognita* show that there are stage-specific differences between surface glycoproteins in this species. Furthermore, differences were also found between the glycoproteins of females for three species of *Meloidogyne*, *M. incognita*, *M. javanica* and *M. hapla*. Further work is required to establish if they have a role in recognition of the parasite by the host.

Keywords: Molecular weight, proteins, glycoproteins, *Meloidogyne incognita*, surface, female.

Introduction

The surface of the nematode is the interface between the worm and its environment. The structure of the cuticle has been studied by electron microscopy [1], but the nature of the surface macromolecules is poorly defined at present. The glycoproteins and proteins on the nematode surface form a glycocalyx, a coat similar to that covering many animal cell surfaces. The cuticle varies in thickness and chemical composition according to cell type and species [2]. Openings to the surface from sense organs, excretory system, mouth and anus may also secrete macromolecules on to the surface of the animal. The glycoproteins associated with such secretions of the nematode provide a binding site for lectin-bearing spores of endozoic, nematophagous fungi [1,3]. The pattern of binding of spores of one lectin-bearing fungus *Verticillium. balanoides* differs among nematode species on this basis [3]. Furthermore, *Ditylenchus dipsaci* populations differ in surface macromolecules recognized by a specific monoclonal antibody probe [4]. Albersheim and Anderson-Prouty [5] reported that the interaction

between host and pathogen is considered to involve the release of endogenous and exogenous elicitors. Endogenous elicitors are of host origin, whereas exogenous elicitors are due to hydrolysis of pathogen components by host enzymes [6, 7]. Such elicitors may establish a wide range of responses involving defence-related proteins and also phytoalexins. The accumulation of the phytoalexin glyceollin I in soybean roots around *Heterodera. glycines* has been considered to be due to release of elicitors [8] and similar effects have been previously reported for *Meloidogyne incognita* [9].

The aim of this experiment was to describe and define the proteins and glycoproteins present on the surface of infective second stage juvenile of *Meloidogyne incognita*. Proteins and glycoproteins were analyzed using samples of whole (non-homogenised) nematodes. Whole nematodes were extracted in sodium dodecyl sulphate(SDS) to release surface proteins. Surface glycoproteins were analyzed by biotinylation of whole worms, followed by application of avidin to nitrocellulose blots of surface glycoconjugates separated by SDS PAGE. The experiments also sought to confirm previous descriptions of the proteins present after homogenisation of *M. incognita*.

Materials and Methods

Sample preparation

Second stage *Meloidogyne incognita* were collected after hatching in distilled water at 25°C. To each volume of settled J2, 0.25 volumes of SDS-sample buffer was added. This buffer consisted of 0.3125 M Tris Hcl, 10% (w/v) SDS, 25% (w/v) glycerol, 0.0125% bromophenol blue, pH 6.8 (to which 50 mM dithiothreitol was added just before use). Two nematode samples were prepared. One sample was homogenised on ice in the buffer using a tissue homogeniser (0.1 ml, Jencons). While the second sample was not homogenised.

Gel preparation

Polyacrylamide gel electrophoresis (PAGE) in the presence of SDS was carried out, essentially to a standard method [10] in gel slabs containing 0.375 M Tris Hcl, pH 8.8, 0.1% (w/v) SDS and 7.5% (w/v) acrylamide, 0.2% (w/v) N,N-methylene-bis-acrylamide, 0.028% (w/v) ammonium persulphate. 0.04% N,N,N-tetramethylethylenediamine (TEMED) was added after degassing with a water-powered vacuum pump. A stacking gel (4.5% acrylamide) 0.125 M Tris, pH 6.8, 0.1% (w/v) SDS, 0.03% (w/v) ammonium persulphate and 0.1% TEMED was added to the top of the resolving gel before use. The gel was transferred to the electrophoresis apparatus and the electrode buffer (25 mM Tris Hcl, 0.192 mM Glycine, 0.1% SDS) was added. The samples were loaded into sample wells using micropipettes (Gilson) and electrophoresis

was carried out. Gels were run at 300V and 50 mA constant current using a power pack (Pharmacia 500/400). High molecular weight standards (HMW) and low molecular weight standards (LMW) were included in slots on each gel. LMW standards were bovine serum albumin (mol wt 66,000D), ovalbumin (mol wt 45,000), glyceraldehyde-3-phosphate hydrogenase (mol wt 36,000), carbonic anhydrase (mol wt 20,100), α-lactalbumin (mol wt 14,200). HMW standards were carbonic anhydrase (mol wt 29,000 D), ovalbumin (mol wt 45,000D), bovine serum albumin (mol wt 66,000 D), phosphorylase B (mol wt 97,400D), β-galactosidase (mol wt 116,000D), myosin (mol wt 205,000D).

Visualisation of proteins

Gels were stained for 1 hour at 37°C in 0.25% (w/v) Coomassie Brilliant blue R 250, 45% methanol, 10% acetic acid. The gel was washed with several changes of destain (25% (w/v) methanol, 10% (w/v) acetic acid) until the background of the gel was colorless. A photograph was taken before the gel was dried. The gel was washed in tap water for 3 h and 5% glycerol was added for 30 min. The gel was removed from the solution, then it was placed between two sheets of cellophane membrane and placed in a Bio-rad Gel lab dryer unit at 60°C for 4 h.

Biotinylation of whole *M. incognita*

Intact J2 *Meloidogyne incognita* were incubated in sodium periodate (50 mM $NaIO_4$ in 0.1 M sodium acetate buffer, pH 4.0) [11] for 30 min on ice, then the J2 *M. incognita* were washed three times with acetate buffer. They were then biotinylated using 10 mM biotin hydrazide in 0.1M sodium acetate buffer, for 15 min on ice. The J2 *M. incognita* were washed four times in phosphate buffered saline (PBS). To test for biotinylation, a subsample of worms was incubated in TRITC-avidin in PBS for one hour at room temperature. They were again washed four times with PBS, transferred to microscope slides and viewed under an epi-fluorescence microscope (Olympus BH-2). Remaining worms were prepared for SDS-PAGE by homogenisation in sample buffer. After electrophoresis the gel was blotted onto nitrocellulose.

Protein blotting

Gels were placed against nitrocellulose in a standard protein blotting tank (Biorad, Trans-Blot TM Cell) and they were immersed in electrophoretic transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol, 0.02% (w/v) SDS). The tank was placed in a cold room (4°C) and electrophoretic transfer carried out overnight at 400 mA.

Visualisation of biotinylated proteins on blots

The nitrocellulose from protein blotting was blocked with PBS containing 1% Tween 20, 1% bovine serum albumin (BSA) for 1 h. Avidin-alkaline phosphatase was applied to nitrocellulose in PBS-Tween 20 for 1 h. It was rinsed with PBS five times for 25 min, then in NBT buffer (0.1 M Tris, 0.1 M NaCl, 5 mM $MgCl_2 6H_2O$) pH 9.3 two times for 10 min each and soaked in substrate (29 mM nitro blue tetrazolium (NBT) and 14.52 mM 5-bromo-4-chloro-3-indolyl phospate (BCIP)). When bands were observed, the nitrocellulose was rinsed in distilled water and dried.

Visualisation of protein standards on protein blots

HMW and LMW standards were treated with amido black stain (0.1% amido black, 25% methanol, 10% acetic acid) for 4 min and several changes of destain used until clear bands were visualised.

Results

Proteins of homogenised juveniles

Major proteins from 3,000 homogenised J2 of *M. incognita* were defined by analysis of SDS-PAGE gels of homogenised nematodes. The main bands of protein detected were at 205, 104, 66, 44 and 33 kD. There were visualised bands in total (Table 1 and Fig. 1A).

The major proteins recovered from surface *M. incognita* incubated in acetate buffer occurred at 205, 104, 44 and 33 kD. In addition, five minor bands were detected between 31 and 44 kD, plus a high molecular weight protein at >205kD (Table 1 and Fig. 1B).

Biotinylation of surface glycoproteins

Fluorescently-labelled avidin was used to check the efficiency of labelling of the surface glycoproteins with biotin and also the distribution of labelling. Fluorescence was seen in both the cuticle surface and anterior region (Fig. 2). Nematodes not exposed to surface biotinylation could not be visualised under the same photographic conditions.

Glycoproteins revealed by biotinylation

Biotinylated glycoproteins were detected at 180, 165, 152, 62 and 51 kD (Table.1). The comparison between the second stage juvenile and female *M. incognita* indicated that there are differences between surface glycoproteins of these two stages (Fig. 3).

Table 1. Molecular weigh of proteins and glycoproteins derived from *M, incognita,* second stage juveniles, whole worm and surface stripped individuals. Glycoproteins of 3 species of *Meloidogyne* females are also given for comparison

Mr x 10^{-3}	Whole nematode proteins	Surface proteins *M. incognita* J2	Surface glycoproteins *M. incognita* J2	Surface glycoproteins of female		
				M. incognita	*M. javanica*	*M. hapla*
>150	2 > 205					
	205	>205	>180			
				180	180	180
			170			
				165	165	165
					162	
				152	152	
100-150	104	104	150			
		145				145
		135				
					130	
					110	
50-100	88	88				
	68	68	74			
	66		66			
		64				
			61	62	62	62
	58	58			59	
		57	-			
			56			
			54			
			51	51	51	51
>50	48		50		49	
	44	44				
	42	42				
		37				
		35	34			
	33					32
	31					

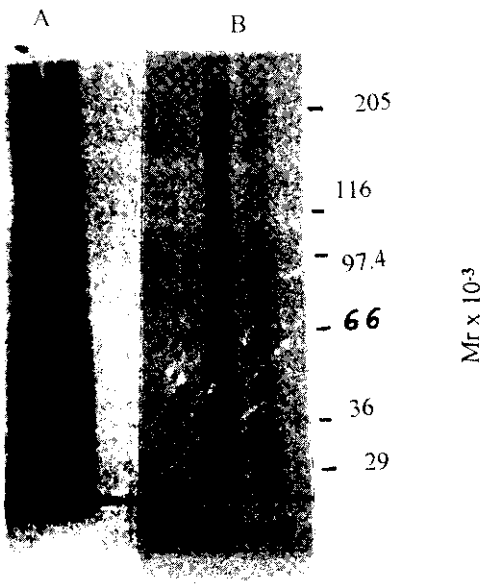

Fig. 1. Polyacrylamide gel analysis of sodium dodecyl sulphate/dithiothreitol soluble (A+B).
B- Whole nematode proteins.
A-Proteins on the surface of second-stage *Meloidogyne incognita*.

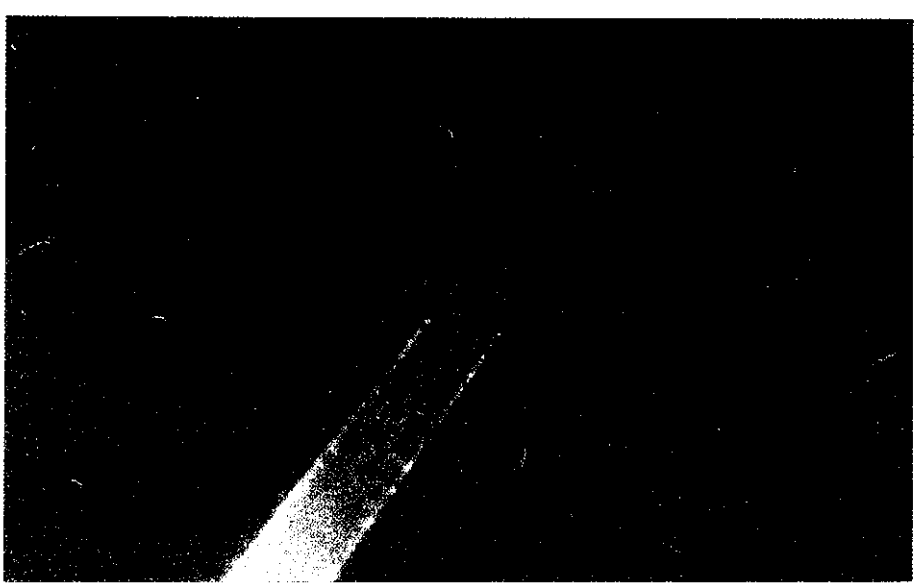

Fig. 2. The efficiency of labelling of the surface glycoproteins with biothin is very strong in both the cuticle surface and samphids. (The arrow shows the anterior face.

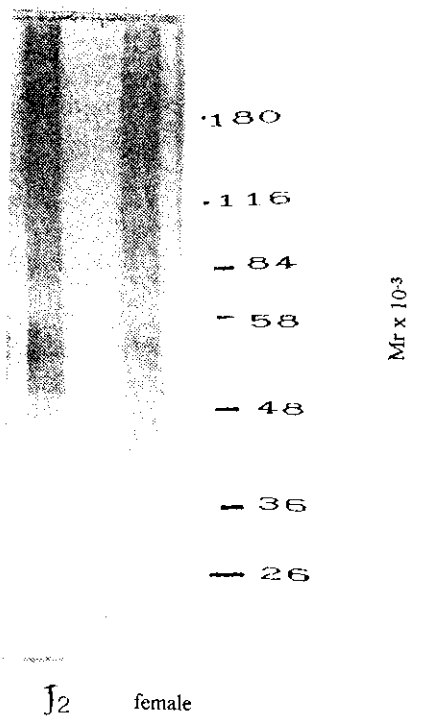

Fig. 3. Surface glycoproteins of second stage and female *Meloidogyne incognita.*

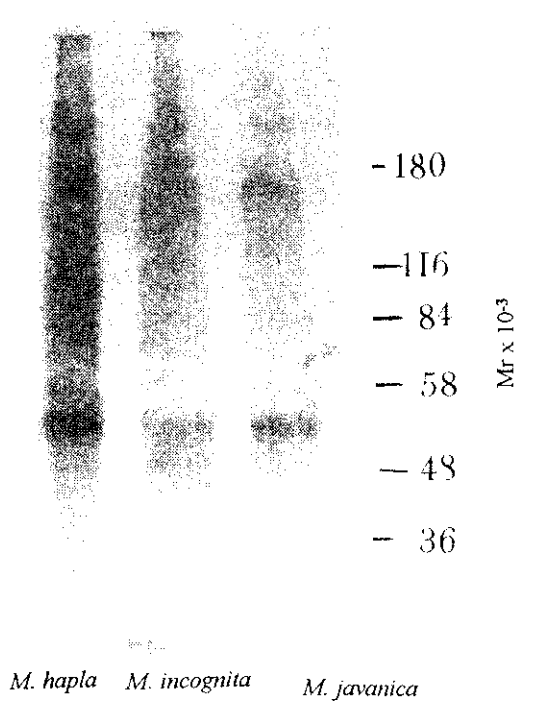

Fig. 4. Surface glycoproteins of *Meloidogyne Incognita Incognita, M. Javanica* and *M. Hapla female.*

Glycoproteins of females of three species of *Meloidogyne*

There are differences between the glycoproteins of *M. incognita, M. javanica* and *M. hapla* females (Fig. 4).

Discussion

The protein loading of gels with whole nematode homogenates was sufficiently low to limit detection to a few of the major proteins. The objective was to determine if any of the bands detected in later work were due to leakage of major proteins from damaged nematodes. A total of 15 proteins of differing molecular weight were detected on the surface of J2 *M. incognita*. Six of the polypeptides detected in the surface extract had similar molecular weight values to those obtained using the whole nematode homogenate, including three major proteins recovered in the later samples at Mr 205, 104 and 44 kD.

The results were broadly similar to those reported by Robinson *et al* [12]. Four major bands of proteins (218, 135, 102 and 43 kD) out of nine reported by Robinson *et al* [12] were found to be superficially located on second stage *M. incognita*. Four main bands of 16 in total of easily extractable proteins were characterised as being associated with the cuticle of second stage juveniles. Robinson *et al* [12] observed 9 bands, not 16 as reported in this work. They used different methods and relied on Bolton-Hunter reagent to catalyse radio-iodination before homogenising the nematodes. They also used two solvents for analysis: sodium deoxycholate and SDS. These differences in technique may explain the difference between their results and those of the current study.

Reddigari *et al* [13] have studied cuticular collagenous proteins of stages of *M. incognita* using a non-intact cuticle preparation. Biochemical analyses of the cuticular components of *M. incognita* were performed using cuticle 'ghosts' in which the nematodes were ruptured and the cuticle separated from internal components by centrifugation [13]. The cuticle of second stage *M. incognita* was observed to be a three-zoned structure consisting of an outer electron-dense cortical zone, a clear median zone with struts and a striated basal fiber zone. The cortical zone was covered by a triple-layered epicuticle. Reddigari *et al* [13] also reported that the cuticle of *M. incognita* contains collagenous proteins that are interlinked to each other and other cuticular components through disulphide bonds. Proteins in the cortical and median zones were partially soluble in β-mercaptoethanol (BME), whereas the basal zone was the least soluble.

The BME-soluble proteins from the juvenile surface were separated into 12 bands. The current work is in general agreement with the previous characterization of the surface proteins of *M. incognita*. Dithiothreitol was used to disrupt disulphide bonds in this case, suggesting that the surface proteins detected were from the cortical and median zones of the cuticle and do not simply represent surface exposed proteins.

Reddigari *et al* [13] observed 9 proteins of different molecular weight in the cuticle of *M. incognita and* Cox *et al* [14] obtained a similar number with the free-living nematode *Caenorhabditis elegans*, while in another free-living nematode, *Panagrellus sillusiae,* [15] 18 or more proteins were observed. The cuticle of *Ascaris suum*, however, contains only 5 or 6 proteins [16,17]. This suggests that considerable differences occur in the surface protein complement of dissimilar nematode species.

Fetterer [18] has studied the cuticular proteins from free-living and parasitic stages of *Haemonchus contortus* using SDS-PAGE to analyze cuticular proteins and improve understanding of the structure and function of the cuticle in adaptation to parasitism. He used a combination of mechanical disruption and detergent treatment. The β-

mercaptoethanol soluble cuticular proteins from adult males contained 4 or 5 major protein bands with molecular weights ranging from 100 to 56 kD with the most prominent band at 56 kD. Cuticular proteins from the juvenile stages, second moult cuticle and third stage cuticle, differed from the adults and from each other. The cuticular proteins from all developmental stages were at least partially digested by bacterial collagenase. The amino acid composition of cuticular proteins was similar for the third stage and second moult, but adults had lesser amounts of glycine and greater amounts of basic amino acids than the larval stages.Fetterer [18] concluded that there were quantitative and qualitative stage-specific differences in the cuticular proteins of *H. contortus*.

The cuticle of J2 *M. incognita* is similar to the cuticle of *Caenorhabditis elegans* (free-living soil nematodes) dauer juvenile in structure and solubility in β-mercaptoethanol [13]. Under adverse environmental conditions, the *C. elegans* J2 develops into a survival stage called the dauer juvenile. Before penetrating the plant, J2 *M. incognita* occurs in soil like many free-living nematodes, but after penetration of a host plant, the internal structure of its cuticle begins to change [13]. Surface proteins may change within one defined life cycle stage [12]. These authors also recognized that the surface protein may be important in the interaction between infective second stage *M. incognita* and the host plant during penetration.

The main bands of glycoproteins on the surface of J2 *M. incognita* were detected in 3 zones,.in addition to 3 other minor bands (Table .1). Other investigators [13,16,19] also studied surface glycoproteins. There were 7 glycoproteins in the BME-soluble fraction from J2 cuticles. In the current work 5 bands of glycoproteins were found in the surface of J2 *M. incognita*. Parkhouse *et al* [19] used a radioactive labelling technique, as did Robinson *et al* [12]. The current investigation used the same technique as Winkfein [16].

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الكشف عن جزيئات البروتين والجليكوبروتين على سطح نيماتودا تعقد الجذور *Meloidogyne incognita*

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(قدم للنشر في ١٤١٦/٧/٧هـ وقبل للنشر في ١٤١٧/٢/١٠هـ)

ملخص البحث – استخدمت طريقة التحليل الكهربائي (SDS - PAGE)للكشف عن جزيئات البروتين والجليكوبروتين الموجود على سطح ومخلوط الطور اليرقي الثاني لنيماتودا تعقد الجذور *Meloidogyne incugnita* أتضح من نتائج التحليل وجود ثلاثة فواصل رئيسية من البروتين على سطح الطور اليرقي الثاني لنيماتودا تعقد الجذور بالإضافه إلى ظهور أحد عشر فاصلاً غير رئيسية . وتم الكشف أيضاً عن جليكوبروتين على سطح الطور اليرقي الثاني للنيماتودا . ومن النتائج أيضاً تبين وجود اختلافات في تركيب الجليكوبروتين السطحي بين الطور اليرقي الثاني وأنثى نيماتودا تعقد الجذور M incognita بالإضافة إلى وجود اختلافات في الجليكوبروتين بين إناث ثلاثة أنواع من نيماتودا تعقد الجذور. *M. hapla , M. javanica , M. incognita*