Towards identification of oesophageal gland proteins in *Globodera rostochiensis*

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Towards identification of oesophageal gland proteins in *Globodera rostochiensis*

Proefschrift

ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen
op gezag van de rector magnificus,
dr. C.M. Karssen,
in het openbaar te verdedigen
op dinsdag 11 juni 1996
des namiddags te half twee in de Aula
van de Landbouwuniversiteit te Wageningen

The work presented in this thesis was performed at the Department of Nematology, Wageningen Agricultural University, Binnenhaven 10, 6709 PD Wageningen, The Netherlands.

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Stellingen

1. Speekseleiwitten van aardappelcysteaaltjes bieden een aangrijpingspunt voor resistentie tegen aardappelmoeheid op basis van 'plantibodies'.

   Dit proefschrift.

2. Het gemak waarmee mannelijke aardappelcysteaaltjes hun lichaamswand-spiermassa opbouwen doet body-builders verbleken.

   Dit proefschrift.

3. De definitie 'moleculaire chaperones zijn een familie van niet verwante klassen van eiwitten' verdient een originaliteitsprijs.

   Ellis, R.J. & Van der Vies, S.M. 1991. Molecular chaperones. *Annual Review of Biochemistry* **60,** 321-347.

4. De produktie van monoklonale antilichamen tegen oppervlakte antigenen van nematoden uit het geslacht *Ditylenchus* is een vorm van hocus-pocus.

   Palmer, H.M., Atkinson, H.J. & Perry, R.N. 1992. Monoclonal antibodies specific to surface expressed antigens of *Ditylenchus dipsaci. Fundamental and Applied Nematology* **15,** 511-515.

5. Het is onwaarschijnlijk dat monoklonale antilichamen nog aan vetdruppeltjes in de darm van de nematode *Heterodera glycines* kunnen binden, nadat deze is behandeld met methanol en aceton.

   Atkinson, H.J. *et al.* 1988. Monoclonal antibodies to the soya bean cyst nematode, *Heterodera glycines. Annals of Applied Biology* **112,** 459-469.

5. De 'zone of modified cytoplasm' die rondom de plaats van stylet-insertie in voedingscellen van plant-parasitaire nematoden wordt waargenomen is waarschijnlijk een 'size-excluding compartment' gevormd door de voedingscel zelf.

   Wyss, U., Stender, C. & Lehmann, H. 1984. Ultrastructure of feeding sites of the cyst nematode *Heterodera schachtii* Schmidt in roots of susceptible and resistant *Raphanus sativus* L. var. *oleiformis* Pers. cultivars. *Physiological Plant Pathology* **25,** 21-37.
   Provance, D.W., McDowall, A., Marko, M. & Luby-Phelps, K. 1993. Cytoarchitecture of size-excluding compartments in living cells. *Journal of Cell Science* **106,** 565-578.

6. Op grond van de Gram-positieve celwandstructuur van *Mobiluncus* is het onjuist deze bacterie in te delen bij de Bacteroidaceae.

   Spiegel, C.A. & Roberts, M. 1984. *Mobiluncus* gen. nov., *Mobiluncus curtisii* sp. nov., *Mobiluncus curtisii* subsp. *holmesii* subsp. nov., and *Mobilucus mulieris* sp. nov., curved rods from the human vagina. *International Journal of Systematic Bacteriology* **34,** 177-184.
   De Boer, J.M. & Plantema, F.H.F. 1988. Ultrastructure of the *in situ* adherence of *Mobiluncus* to vaginal epithelial cells. *Canadian Journal of Microbiology* **34,** 757-766.

7. Elektroforese is een celbiologisch proces.

   De Loof, A. 1986. The electrical dimension of cells: the cell as a miniature electrophoresis chamber. *International Review of Cytology* **104,** 251-352.

9. In consumptie-ijs wordt de 'heat shock response' gereguleerd door dextranen.

McCurdy, R.D., Goff, H.D. & Stanley, D.W. 1994. Properties of dextran as a cryoprotectant in ice cream. *Food Hydrocolloids* **8**, 625-633.

10. Het is onjuist dat elektronen-doorzichtige blaasjes in met osmiumtetroxide gefixeerde eicellen lipiden bevatten.

Bleve-Zacheo, T., Melillo, M.T. & Zacheo, G. 1993. Ultrastructural studies on the nematode *Xiphinema diversicaudatum*: oogenesis and fertilization. *Tissue Cell* **25**, 375-388.
Hayat, M.A. 1981. *Fixation for electron microscopy.* New York: Academic Press. (p. 7).

11. Afwezigheid van zelfcorrelatie in pseudo-toevalsgetal generatoren kan via grafische tests slechts worden vastgesteld indien men wacht totdat het beeldscherm *geheel* vol geplot is.

Chiu, T.-W. & Guu, T.-S. 1987. A shift-register sequence random number generator implemented on the microcomputers with 8088/8086 and 8087. *Computer Physics Communications* **47**, 105-117.
Hamilton, K.G. 1993. Pseudorandom number generators for personal computers. *Computer Physics Communications* **75**, 105-117.

12. Lieveheersbeestjes?

Hurst, G.D.D. *et al.* 1995. Sexually transmitted disease in a promiscuous insect, *Adalia bipunctata*. *Ecological Entomology* **20**, 230-236.

13. Door voortgaande specialisatie van het onderzoek zullen molekulair biologische publikaties verworden tot hindernisbanen van acronymen en afkortingen.

Wang, X.W. *et al.* 1995. p53 modulation of TFIIH-associated nucleotide excision repair activity. *Nature Genetics* **10**, 188-195.

14. Het is verbazingwekkend dat in het huidige informatie- en hobbytijdperk de term 'computersport' nog niet is ingeburgerd.

Stellingen behorend bij het proefschrift getiteld 'Towards identification of oesophageal gland proteins in *Globodera rostochiensis*' door J.M. de Boer.

Wageningen, 11 juni 1996.

Contents

Chapter 1

General introduction

Pests of potato

Potato cyst-nematodes (*Globodera rostochiensis* and *G. pallida*) are obligate root parasites of solanaceous host plants, which affect potato cultivation world-wide (Evans & Stone, 1977). In the Netherlands, infestations with potato cyst-nematodes (PCN) are wide-spread and the acreage of arable land infested with PCN is still increasing (Anonymus, 1994; Molendijk, 1991). Frequent growth of potato on the same field requires the use of control measures to avoid a build-up of the nematode population in the soil to levels where economic damage occurs. In the Netherlands, these control schemes are a combination of crop rotation, the use of resistant potato varieties, and soil fumigation with nematicides. While resistant potatoe varieties can be applied reasonably effective to control *G. rostochiensis*, this is not the case for *G. pallida* because few resistant cultivars are available against this species (Anonymus, 1994; Anonymus, 1995). It is for this reason that *G. pallida* has already become the predominant species of PCN in the starch potato growing areas (Mulder, 1994), and also in other parts of the Netherlands *G. rostochiensis* is being replaced by *G. pallida* (Anonymus, 1994). One therefore still has to rely much on the use of nematicides to control PCN. This, however, will become more and more difficult because the Dutch government enforces a progressive reduction in the use of nematicides towards the year 2000 (Anonymus, 1994). It is therefore expected that the near future wil bring an urgent need for more potato varieties that are (partially) resistant to PCN.

Rationale of the thesis

In the early 1980's a research programme was started at the Department of Nematology with the aim to acquire more information about the genetics and molecular biology of the interaction between PCN and their host plant. The emphasis was put on the nematode side of this interaction, and on *G. rostochiensis* in particular because monogenic resistance in potato was available only to this species at that time. To start with, virulence characteristics of populations of PCN have been compared with two-dimensional electrophoresis patterns

of proteins (Bakker, 1987), and patient genetic crossings have demonstrated a gene-for-gene relationship between a virulence gene in *G. rostochiensis* and the H1 resistance gene in potato (Janssen, 1990). Furthermore, monoclonal antibodies have found their application in an immunoassay which discriminates between both species of PCN (Schots, 1988). The present thesis is the logical follow-up of this preceeding work. Its goal was to identify the secretory proteins of the oesophageal glands of *G. rostochiensis*. These secretory proteins are assumed to be involved in the interaction with the host plant, and they may be the factors determining virulence in PCN and other cyst-nematodes. It was anticipated that knowledge of these "saliva proteins" would ultimately find its use in the introduction of novel forms of (artificial) resistance to PCN in potato. As outlined in the preceeding paragraph, the arrival of such new resistance forms will be more than welcome in the years to come.

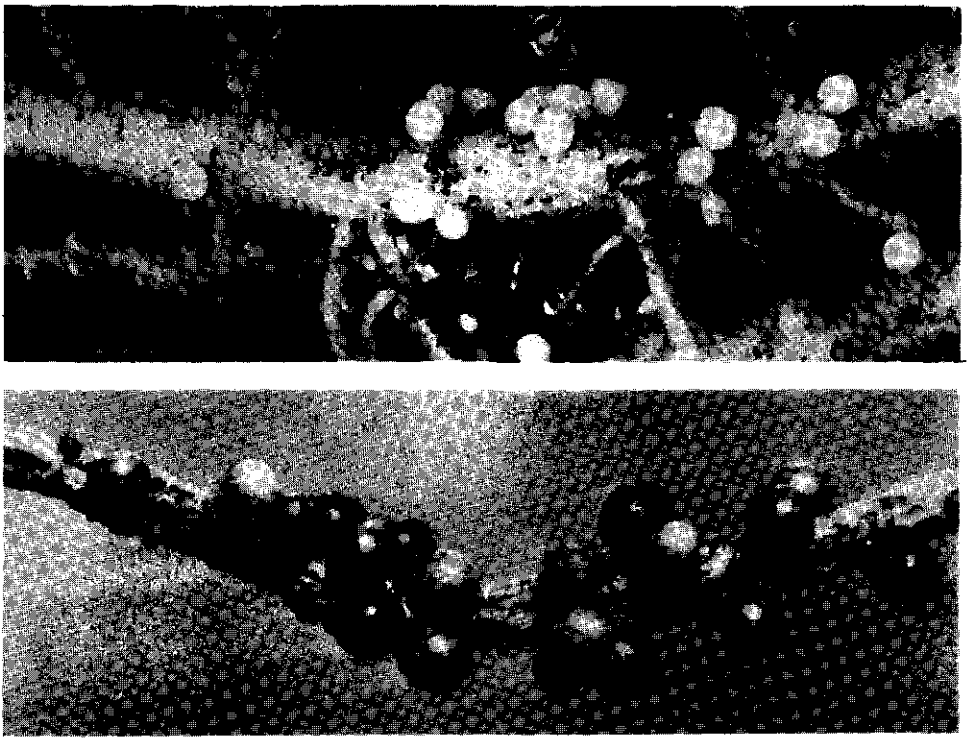

Fig. 1.1. Details of potato roots covered with *Globodera rostochiensis*. **Above:** Young full-grown females have broken through the root surface and are visible as white globules. **Below:** When the females die, they gradually attain a dark brown colour and become cysts. Each cyst contains a few hundred eggs with infective juvenile nematodes in diapause (From: Spears, 1968).

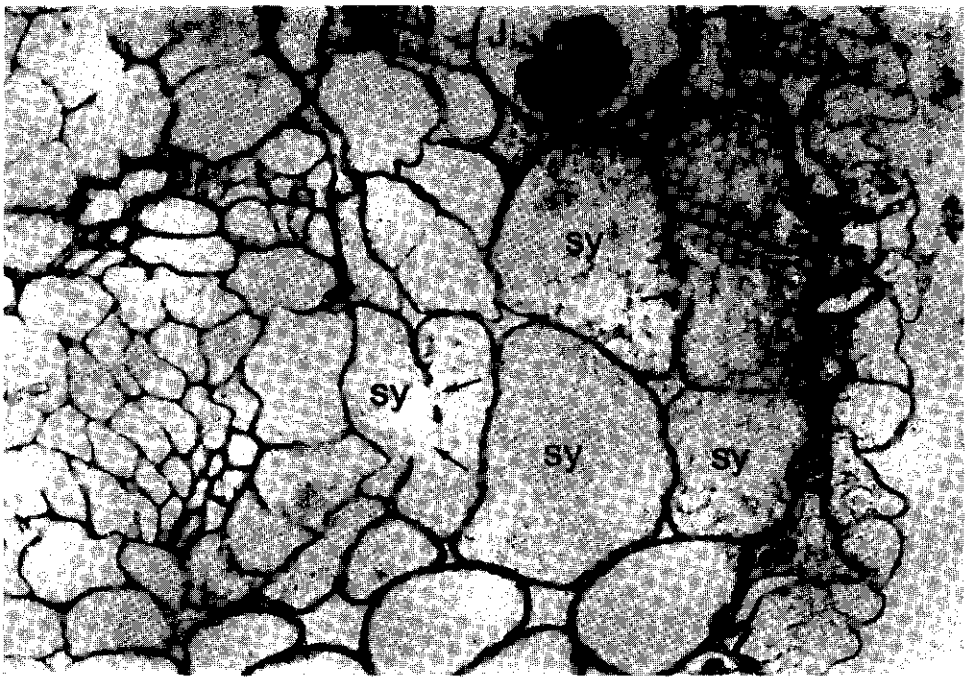

Fig. 1.2. Early syncytium induced by a juvenile (J) of *Globodera rostochiensis* in a rootlet of potato cv. Bintje. The syncytium (Sy) is located in the cortex and shows fragmentation of the vacuoles and dissolution of cell walls (arrows). (Photograph by Hein Overmars).

Saliva proteins

When phytonematologists speak of 'saliva proteins', 'speeksel', '(o)esophageal gland secretions', 'pharyngeal gland secretions', 'spit', 'stylet secretions', 'protéines salivaires' or 'Speicheldrüsensekret', they commonly refer to an elusive set of secretory products, which are produced by three unicellular glands that are found in cyst-nematodes and other plant-parasitic nematodes of the order Tylenchida. These oesphageal gland secretions have been the subject of study and speculation for more than three decades because they are considered to play an essential role in the feeding relationship between these nematodes and their host plant (Hussey, 1989).

Cyst-nematodes feed from a highly specialized syncytium of modified root cells which is induced by the infective second-stage juvenile after its entry into a root of the host plant (Fig. 1.2). With its mouth stylet (a hollow needle) the juvenile pierces the cell wall of this syncytium and withdraws its fluid contents for nourishment. The syncytium presumably functions as a transfer cell by passing

nutrients from the adjacent vascular tissue to the feeding nematode (Jones & Northcode, 1972; Gunning, 1977). The nematodes are entirely dependent on this feeding cell for their growth and development and if it malfunctions or disintegrates prematurely, as is for instance the case in resistant potato varieties (Hoopes *et al.*, 1978; Rice *et al.*, 1985; Rice *et al.*, 1987), then they will die.

The ability of cyst-nematodes to induce syncytia within the roots of their host plant is remarkable, and is shared only with certain other groups of sedentary plant-parasitic nematodes which also exploit feeding cells. Second stage juveniles of PCN which have penetrated the root start feeding from a single cell, which is usually located in the root cortex. Following stylet insertion by the juvenile, this vacuolated cell soon transforms into a metabolically activated cell, in which the vacuole disintegrates and cell organelles proliferate. Cell walls are digested at the pit fields and fusion of neighbouring cell protoplasts causes the formation of a multinucleate syncytium (Fig. 1.2; Jones & Northcote, 1972; Steinbach, 1973; Hoopes *et al.*, 1978; Jones, 1981; Melillo *et al*, 1990). Where the syncytium contacts vascular tissue, there is often a deposition of cell wall ingrowths of a type that is also seen in transfer cells (Jones & Northcote, 1972; Rice *et al.*, 1985; Gunning, 1977). In syncytia induced by the beet cyst-nematode a cell cycle gene promoter is activated already in a very early stage (Niebel *et al.*, 1994), while several other promoters show downregulation (Goddijn *et al.*, 1993). This syndrome of events leading to syncytium formation is not matched by any naturally occurring cell type or event in plant development, and can therefore be considered a host plant response that is unique to nematode infection. This leaves, however, a fundamental questions unanswered: how do the nematodes induce this plant response?

Following root penetration, the juvenile cyst-nematodes must somehow signal to a mature root cell that it has to undergo transdifferentiation into a feeding cell. An early hypothesis was that simply the withdrawal of cytoplasm by the feeding nematode would initiate syncytium formation: the nematode being a metabolic sink that induces a local response in the root tissue in the form of a syncytium (Jones & Northcote, 1972; Bird, 1979). It was recently demonstrated, however, that the patatin class I promoter, which is an indicator of sink cells in plants, was not active in syncytia induced by the beet cyst-nematode (Goddijn *et al.*, 1993). It is therefore more likely that other factors of nematode origin cause syncytium formation. It is now generally assumed that stylet secretions originating from the oesophageal glands are responsible for syncytium induction: by injecting these secretions into the cytoplasm of a root cell, the juvenile nematodes may introduce the necessary signals to evoke a feeding cell response by the host plant (Rice *et al.*, 1985; Rice *et al.*, 1987; Wyss & Zunke, 1986; Hussey, 1989).

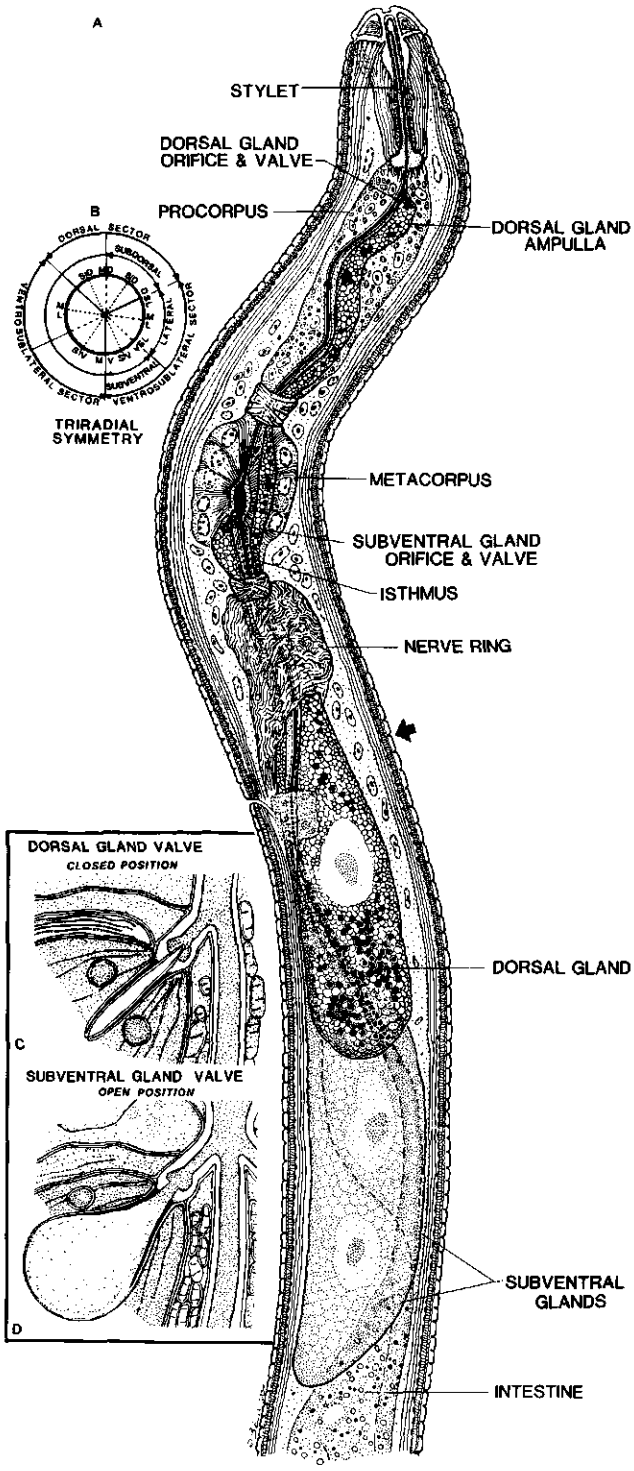

Fig. 1.3. Anterior part of a 2nd-stage infective juvenile of a cyst-nematode (From: Endo, 1984).

The saliva proteins of cyst-nematodes are synthesized in one dorsal and two subventral oesophageal gland cells (Fig. 1.3), where they are stored in secretory granules (Fig. 1.4). These granules are transported anteriorly into a narrow extension of the gland cell and they release their contents into the oesophageal lumen via a valve that is situated at the end of this extension. The secretory products of both glands normally leave the nematode via the stylet (Wyss, 1992; Goverse *et al.,* 1994), although indications exist that some products of the subventral glands may be transported to the intestine (Wyss, 1992). In infective second stage juveniles the three glands are approximately equal in size (Fig. 1.3). However, as soon as the juveniles have entered the root and established a feeding site, the dorsal gland becomes larger (Fig. 1.5) and increases its activity, whereas the subventral glands undergo a reduction in size which is related to a change in the nature of their secretory products (Wyss, 1992; Endo, 1987; Endo, 1993).

Despite many years of study on cyst-nematodes there is still very little hard evidence concerning the function of their oesophageal gland secretions. Video microscopic observations of feeding beet cyst-nematodes have conclusively demon-

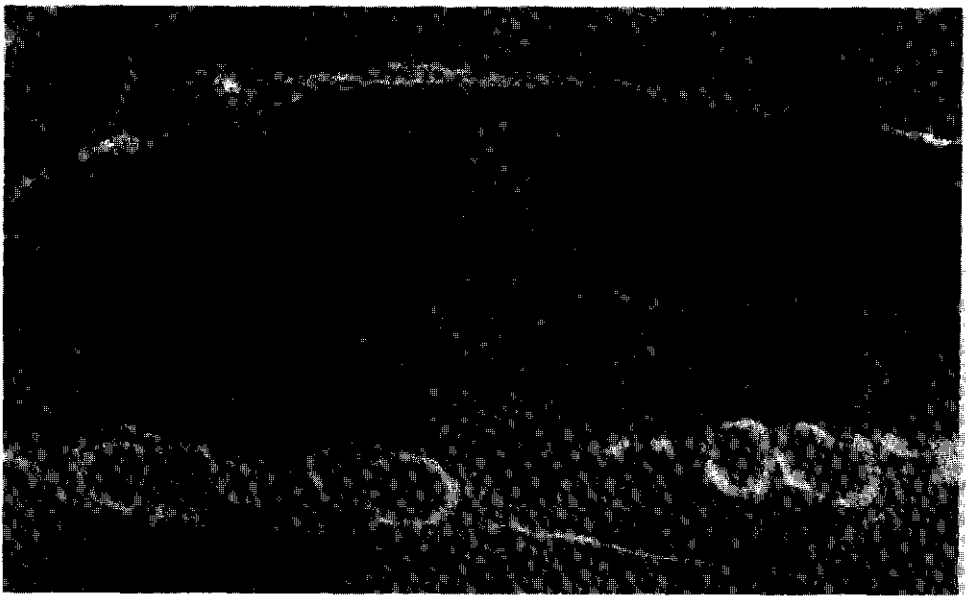

Fig. 1.4. Electron micrograph of the oesophageal gland region of an infective J2 of *G. rostochiensis* (oblique section; approximate position indicated with arrow in Fig. 1.3). The anterior part of the dorsal gland cell body is filled with small secretory granules (dg). Also visible are the extensions of both subventral glands which contain secretory granules of a larger diameter (svg). ol = triradiate oesophageal lumen. Bar = 1 μm.

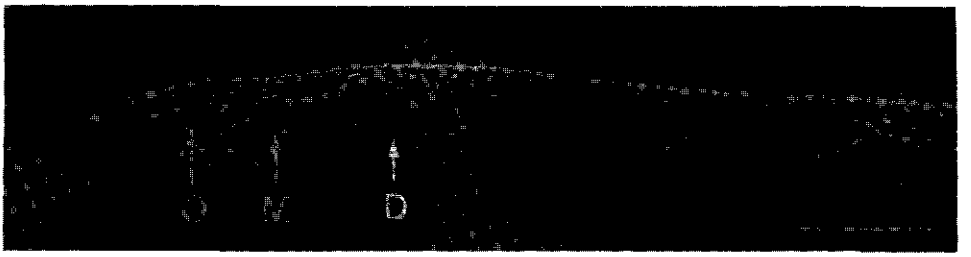

Fig. 1.5. Parasitic 2nd-stage juvenile of *G. rostochiensis* showing the enlarged dorsal oesophageal gland (D), the metacorpus (M), and the anterior oesophagus (O). Interference contrast illumination. Bar = 5 μm.

strated that secretions from the dorsal gland are responsible for the formation of feeding tubes within the syncytium (Wyss & Zunke, 1986; Wyss, 1992). Feeding tubes have been observed in feeding cells of many plant parasitic nematode species, including PCN (Rumpenhorst, 1984; Melillo *et al.* 1990), and it is assumed that these structures function as molecular sieves during food uptake (Böckenhoff & Grundler, 1994). In addition, various other functions have been ascribed to the oesophageal gland secretions, but these remain as yet unproven. Apart from the possible role that the gland secretions have in syncytium induction, it has been proposed that they are necessary for maintenance of the syncytium (Hussey, 1989), that they digest cell walls during root penetration (Steinbach, 1972), that they modify or predigest the zone of cytoplasm surrounding the feeding site (Wyss *et al.*, 1984; Wyss & Zunke, 1986), that they prepare the intestine of the juveniles for food uptake and assist in the mobilization of intestinal lipid reserves (Wyss & Grundler, 1992; Atkinson & Harris, 1989), and that they produce enzymes for intestinal food digestion (Wyss, 1992). Although at first sight it seems unlikely that this many functions can be taken care of by only two gland types, it is very well possible that developmental changes occur in the nature of their secretory products, as has already been noticed for the subventral glands of *Heterodera schachtii* (Wyss, 1992) and *H. glycines* (Endo, 1993).

Outline of the thesis

This thesis presents the data which were collected during a search for oesophageal gland proteins in *G. rostochiensis.*

The first step of this search was a comparison of silver-stained two-dimensional protein patterns from developmental stages of *G. rostochiensis.* This analysis is presented in Chapter 2. It was thought that a comparison between parasitic

and non-parasitic stages would single out a few proteins as potential candidates for the secretory proteins. This turned out to be a misconception. The protein variation between the developmental stages was large, and on top of this the applied electrophoresis technique was not sensitive enough to analyse protein samples from the smallest (but most important) parasitic juvenile stages.

We therefore developed a sensitive miniature 2D-electrophoresis technique, which is presented in Chapter 3. The sensitivity of the protein silver stain was optimized using the genotyping of single females of *G. pallida* as a model system.

In Chapter 4 this mini-2D electrophoresis technique is applied for protein analysis of the complete range of post-embryonic developmental stages of *G. rostochiensis*, including the earliest parasitic juvenile stages. However, exactly at the onset of parasitism, where changes in expression of gland proteins can be expected to occur, the changes in the protein patterns were again far too large to make a sensible selection of protein spots for closer analysis.

In Chapter 5 we applied microdissection to live J2 and males of *G. rostochiensis* with the aim to identify oesophageal gland proteins. Protein samples from front parts and rear parts were analysed using mini-2D electrophoresis. This identified a single protein spot which occurred specifically in the front part of both J2 and males, and which could therefore be an oesophageal gland protein. However, later immunizations with excised gel segments containing this protein spot failed to produce antibodies reactive with the oesophageal glands, and its precise origin is still unresolved.

In Chapter 6 a different approach is started with the production of monoclonal antibodies (MAbs) to J2 of *G. rostochiensis*.. Five MAbs were obtained which reacted specifically with the subventral oesophageal glands of J2 in immunofluorescence assays. Unfortunately these MAbs did not detect saliva proteins in electrophoresis patterns of J2, presumably because their epitopes were damaged by the electrophoresis detergent sodium dodecyl sulfate (SDS).

In Chapter 7 we therefore tried a novel method of antigen preparation for antibody production: proteins from homogenized J2 were fractionated by preparative electrophoresis in the presence of SDS and used for immunization. This resulted in a new panel of MAbs specific to the subventral glands. In contrast to the MAbs of Chapter 6, these MAbs were directed to epitopes that resisted denaturation by SDS, and this led to the identification of 4 subventral gland-specific proteins on Western blots of J2. Finally, it is discussed in Chapter 7 that the MAbs to these oesophageal gland proteins may be useful for engineering resistance to PCN in potato.

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Chapter 2

Analysis of two-dimensional protein patterns from developmental stages of the potato cyst-nematode, *Globodera rostochiensis*

Extended version of: De Boer, J. M., Overmars, H. A., Bakker, J. & Gommers, F. J. 1992. Parasitology, 105, 461-474.

Summary

Two-dimensional polyacrylamide gel electrophoresis was used to examine the differences in total protein composition between two motile stages and two sedentary stages of the potato cyst-nematode, *Globodera rostochiensis*. Using a sensitive silver stain, 542 reproducible protein spots were distinguished. A list of these spots is presented, showing their apparent molecular weights, estimated isoelectric points, and occurrences in the different developmental stages. When the protein patterns were compared, 401 spots were found to change their presence or size in one or more of the four developmental stages. It is therefore estimated that during the post-embryonic development of *G. rostochiensis* 74% of the polypeptides undergo modulation of their synthesis, or are affected by protein degradation or modification. In the motile stages several abundant proteins were present, which disappeared or decreased in concentration in the sedentary stages. Some of these proteins are presumably muscle proteins, and their modulation may illustrate the degeneration of body-wall musculature in the sedentary stages. It is concluded that the potato cyst-nematode has a very dynamic protein metabolism.

Key words: development, protein metabolism, body-wall muscle, electrophoresis

Introduction

Cyst-nematodes (genera *Globodera* and *Heterodera*) are highly specialized plant parasites that complete their development almost entirely inside the roots of their host. Their life cycle is complex, and includes both motile non-feeding stages and sedentary parasitic stages which feed by maintaining and exploiting a syncytium of modified root cells (Chitwood & Buhrer, 1946; Raski, 1950; Jones & Northcote, 1972; Evans & Stone, 1977). The active phase of cyst-nematode development begins with hatching of 2nd-stage juveniles from cysts in the soil. A juvenile enters the roots of a host plant and migrates intracellularly to the central vascular cylinder where it induces formation of a feeding-cell complex in the form of a syncytium. These syncytia pass nutrients from the root's transport vessels to juvenile nematodes, and are essential for their growth and development. As soon as juveniles start feeding from their syncytia, they break down their body-wall muscles (Günther, 1972; Shepherd & Clark, 1978) and transform into sedentary parasites. Male juveniles feed only for a few days until the third moult before undergoing a second metamorphosis during which they mature to adults. During this transformation the body-wall musculature is restored, and adult males that leave the roots are fully motile again. The development of females is mainly characterized by a large increase in body weight due to extensive growth of the ovaries and the associated production of eggs. The female starts to swell during the fourth juvenile stage and this growth persists during early adulthood, until the nematodes have become globe (*Globodera*) or lemon (*Heterodera*) shaped. After fertilization, the eggs develop within the female's body as far as the second juvenile stage. Then the females die, and their cuticles tan to form a protective cyst, each containing several hundred eggs with nematodes in diapause.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) has been used to examine developmental changes in protein expression in several organisms, such as a slime mold (Cardelli *et al.*, 1985), an insect (Summers *et al.*, 1986), a free-living nematode (Johnson & Hirsh, 1979), an amphibian (Crise Mueler & Malacinski, 1985), the chicken (Lovell-Badge *et al.*, 1985) and the carrot (Racusen & Schiavone, 1988). Application of 2D-PAGE to cyst-nematodes has been limited mainly to taxonomic or genetic studies in which usually only a single developmental stage was examined (Ohms & Heinicke, 1985; Ferris *et al.*, 1986; Bakker & Bouwman-Smits, 1988). Information about the variation in protein expression during development of these economically important crop pests is therefore still sparse. In this paper we have used 2D-PAGE to map the post-embryonic development of the potato cyst-nematode *Globodera rostochiensis*. Because of the small size of this endoparasite, it was not feasible to obtain sufficient quantities of all of its developmental stages. We therefore limited our analysis to the four stages that were easiest to collect. Two-dimensional protein

patterns were made from 2nd-stage juveniles (J2), adult males, 4th-stage female juveniles (J4F), and adult females. These patterns have been used to provide a detailed comparison of the protein composition of two motile (J2 and adult male) and two sedentary stages (J4F and adult female) of *G. rostochiensis.*

Materials and methods

Chemicals

Acrylamide, *bis*-acrylamide, Tris, glycine, ampholytes, urea, 2-mercaptoethanol, SDS, Coomassie brilliant blue G-250, and molecular weight markers were from Bio-Rad Laboratories (Hercules, USA). Nonidet-P40 was from Sigma Chemical Co. (St. Louis, USA). Silver nitrate and citric acid were from BDH Chemicals Ltd. (Poole, England). All other chemicals were from Merck (Darmstadt, Germany). Distilled, deionized water was used throughout for preparing the solutions and for the washing steps of the silver stain.

Nematodes

Population Ro1-mier of *G. rostochiensis* was grown on *Solanum tuberosum* ssp. *tuberosum* cv. Eigenheimer. Clay pots of 1 litre vol were filled with sandy loam supplemented with 2 g of a slow release fertilizer and inoculated with 200–400 cysts and two seed tubers. The plants were grown at 18 °C with 16 h light/day.

The 2nd-stage juveniles were hatched from cysts that had passed the diapause period, by soaking them in potato root diffusate (Clarke & Perry, 1977). The juveniles were collected daily for approximately 1 week and stored at 4°C. Viable juveniles were allowed to migrate overnight through a cotton wool filter. For each sample 50000–100000 individuals were concentrated by centrifugation at 1000 *g*, and washed twice in 10 mM Tris-HCl, pH 7.4.

To obtain adult males, 4th-stage parasitic juveniles were isolated from infected plants 4–5 weeks post-inoculation. The rootballs were washed thoroughly and cut into 1 cm pieces. The root segments were suspended in water and homogenized in a blender for 30 s. The homogenate was sieved and the 100–250 μm fraction was collected. This suspension was further purified from excess root fragments by centrifugation in 35% (w/v) sucrose for 5 min at 1000 *g*. The floating nematodes were then transferred to tap water and poured out over a cotton wool filter. The 4th-stage males were allowed to complete their development on the filter, and viable adults migrating through it were collected daily for about 1 week. For each sample about 5000 adult males were concentrated by centrifugation (1000 *g*) and washed twice in 10 mM Tris-HCl, pH 7.4.

To obtain 4th-stage females, a purified nematode suspension was prepared from homogenized roots as described for males. For each sample 500–1000 J4F were handpicked from this suspension under a dissecting microscope, and washed

twice in 10 mM Tris-HCl, pH 7.4.

Females were harvested 6–7 weeks post-inoculation. They were washed off rootballs and collected on a 250 μm sieve. After centrifugation (1000 *g*) in . (w/v) sucrose to remove remaining sand and debris, the floating nematodes v transferred to tap water. For each sample about 300 full-grown, white fem were handpicked under a dissecting microscope, and washed twice with 10 . Tris-HCl, pH 7.4.

Sample preparation

Total protein extracts were prepared by homogenizing the nematodes i small glass mortar at 4 °C in 30 μl of 10 mM Tris-HCl, pH 7.4, supplemer with 5% (v/v) 2-mercaptoethanol. After homogenization, another 30 μl of buffer was added, and the samples were saturated with 64 mg of urea. All prot samples were stored at -80 °C until used. A mixture (13 μl) containing 10% (' Nonidet-P40, 25% (v/v) 2-mercaptoethanol, 8% (w/v) ampholytes pH 5–7, and (w/v) ampholytes pH 3–10, was added to the thawed sample before elec phoresis. Prior to application, the samples were spun for 20 min at 30 (100 000 *g*) in an air-driven centrifuge (Beckman Instruments Inc, Palo A USA) to sediment undissolved material. Protein determinations were m according to Bradford (1976) using bovine serum albumin as a standard. isoelectric focusing (see below) 50 μg of protein were applied/gel. This quan corresponds to approximately 9200 J2, 540 males, 91 J4F or 34 females.

Gel electrophoresis

Two-dimensional gel electrophoresis was performed essentially according the method of O'Farrell (1975) with the modifications of Bakker & Bouwm Smits (1988). The isoelectric focusing gels were 4% acrylamide and contain 1.6% ampholytes pH 5–7, 0.4% ampholytes pH 3–10, 9.15 M urea and Nonidet-P40. They were polymerized in glass tubes of 16 cm length and 2 r inner diameter, with 2.5 cm space on top for sample application. Focusing v performed at room temperature without pre-run using a Protean II Tube C (Bio-Rad, Hercules, USA) with the following voltage schedule: 6 h 30 V, 30 r 100 V, 30 min 200 V, 11 h 300 V, and 3 h 400 V. Second-dimension SDS-elect phoresis was performed with a Protean II Multi-Cell (Bio-Rad, Hercules, US. Separation gels were 160 × 135 × 1.5 mm (width × height × thickness) with 1 acrylamide. Stacking gels were 4% acrylamide. The focusing gels were moun on the stacking gel with the aid of melted agarose solution. The upper (ano electrode buffer was 67 mM Tris, 0.25% SDS, and 693 mM glycine, while lower reservoir contained a 2.5-fold dilution of this buffer. Electrophoresis v carried out for 5–7 h at room temperature using 30 mA/gel. Ammoniacal sil staining of the gels was according to Oakley *et al.* (1980), as adapted by Bak

& Bouwman-Smits (1988), with the modification that the rinsing step after the glutaraldehyde incubation was shortened to 4 changes in distilled water of 5, 15, 60, and 120 min respectively.

Molecular weights

The apparent molecular weights of the separated proteins were estimated using the reference proteins myosin heavy chain (200 kDa), beta-galactosidase (116 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21.5 kDa). The focusing gels did not span the full width of the separating gels, and molecular weight markers were therefore applied to a slot in the agarose layer at the end of the focusing gel. A calibration graph was constructed of the logarithm of the molecular weight versus the migration distance, and the molecular weights of the separated proteins were calculated from their migration distance by linear interpolation between subsequent pairs of marker proteins. Molecular masses of less than 21.5 kDa were estimated by extrapolation from the bottom two marker proteins. The molecular weights were not calculated for spots that migrated slower than the myosin heavy chain marker.

Estimation of isoelectric points

Two isoelectric focusing tube gels were cut into 1 cm pieces, and pairs of corresponding gel segments were homogenized in 1 ml of degassed water. The pH of the homogenates was measured at room temperature using a miniature electrode, and a graph was constructed of the pH value versus the migration distance. The focusing pH values of the separated proteins were calculated from their migration distance by linear interpolation between adjacent pairs of pH measurements, and these values were used as estimates of their isoelectric points.

Examination of gels

The protein patterns were compared by superimposing the original gels on a bench viewer. Spots were scored as present in a given stage only if they were also visible in one or more duplicate gels. If the presence or absence of a spot could not be determined with certainty in a given stage, e.g. due to fusion with other spots, such a spot was omitted from the analysis.

With a scale loupe duplicate measurements were taken of the width and height of each spot in each stage. These spot measurements were usually done on two series of well resolved gels. If necessary, other gels were consulted. Using the ellipse as an approximation of the spot shape, the duplicate spot measurements were combined to calculate an average spot area for each stage. On the basis of this area the spots were (arbitrarily) assigned size classes, with 'small' being ≤ 2 mm^2, 'medium' being > 2 and ≤ 5 mm^2, 'large' being > 5 and ≤ 10 mm^2, and

'extra large' being >10 mm^2. In the text these size classes are abbreviated to S, M, L, and XL respectively.

Similarity indices between protein patterns were calculated according to Aquadro & Avise (1981) using the formula $2n_{xy}/(n_x + n_y)$, with n_x and n_y being the numbers of spots observed in stages X and Y respectively, and n_{xy} the number of spots common to both stages. To account for any size differences in shared spots, only those spots that did not show a significant ($P>0.05$) difference in area between the two stages were scored as common spots. For this the Student's t-test was applied to the duplicate spot size measurements.

To assess the number of spots that showed little or no size variation throughout the stages examined, the spots that were common to all four stages were selected, and the difference between their minimum and maximum area in any of the stages was tested with the Student's t-test for lack of significance ($P>0.05$).

The assumption that the variation in the size of a silver-stained protein spot in different gels of a given developmental stage can be described with a normal distribution, was verified by analysing the size distribution of 38 spots in 2D-gels of females with the Kolmogorov-Smirnov test for goodness of fit (Sokal & Rohlf, 1981). The number of gels examined/spot ranged from 7 to 12. It was found that of 35 spots the size distribution did not deviate significantly ($P\leq0.05$) from a normal distribution. Hence, it was concluded that no major errors had been made by comparing spot size differences with the Student's t-test.

Results

General spot counts

In the 2D-PAGE gels of the 4 stages of potato cyst-nematode examined a total of 542 reproducible protein spots could be distinguished with the silver stain. A list of these spots with their variation in size, apparent molecular weight and estimated isoelectric point is shown in Fig. 2.1. The positions of these spots in the two-dimensional protein patterns are shown in Figs. 2.2 to 2.5.

The total number of spots/stage (Table 2.1) ranged from 330 in males to 376 in J2 and the majority were small or medium sized. Large and extra large spots were much less frequent, and ranged from 72 (19.1%) in J2 to only 38 (10.7%) in J4F. The number of stage-specific spots (Table 2.1), i.e. spots visible in only one of the stages examined, varied from 29 (8.8%) in males to 48 (12.8%) in J2. Most of them were also small or medium sized. Extra large stage-specific spots were found only in males.

Protein variation

The number of invariant protein spots was determined from the number of ubiquitous spots. It was found that 210 spots (38.7%) were consistently present in

all four developmental stages. Since many of these spots still showed considerable variation in size, a further selection was made for spots with no significant differences in size among the stages examined. This resulted in a group of only 141 (26%) invariant proteins, of which the concentration in the sample homogenates appeared to be little affected by the course of development. Some of the major invariant spots are labelled with large open arrows in Figs. 2.6 to 2.9. In Fig. 2.1 the invariant spots are marked with an asterisk.

The number of variant proteins was determined by subtracting the number of invariant spots from the total number of spots. This resulted in a group of 401 (74%) polypeptides, whose presence or spot dimensions had changed between the stages examined.

(the text continues on page 34)

Table 2.1. Counts of protein spots visible in two-dimensional electrophoresis patterns of *Globodera rostochiensis*. (Counts were made of all spots, and also of spots that were unique to a given developmental stage. Separate counts were made for the spot size classes S, M, L and XL. J2, 2nd-stage juveniles; J4F, 4th-stage female juveniles.)

		Number of spots				
Stage	Count	Total	S	M	L	XL
J2	All	376	192	112	45	27
Males	All	330	162	104	34	30
J4F	All	353	205	110	27	11
Females	All	367	224	100	33	10
J2	Unique	48	29	14	5	0
Males	Unique	29	7	12	6	4
J4F	Unique	32	22	8	2	0
Females	Unique	31	19	5	7	0

Fig. 2.1 (on next two pages). List of reproducible spots visible in silver-stained two-dimensional protein patterns of the potato cyst-nematode *Globodera rostochiensis*. For each spot its apparent molecular mass in kDa (MW) and focusing pH (pI) are given, together with a graphic representation of its average size in the developmental stages examined. Invariant spots are marked with an asterisk. J2, 2nd-stage juveniles; Ma, males; J4F, 4th-stage female juveniles; Fe, females.

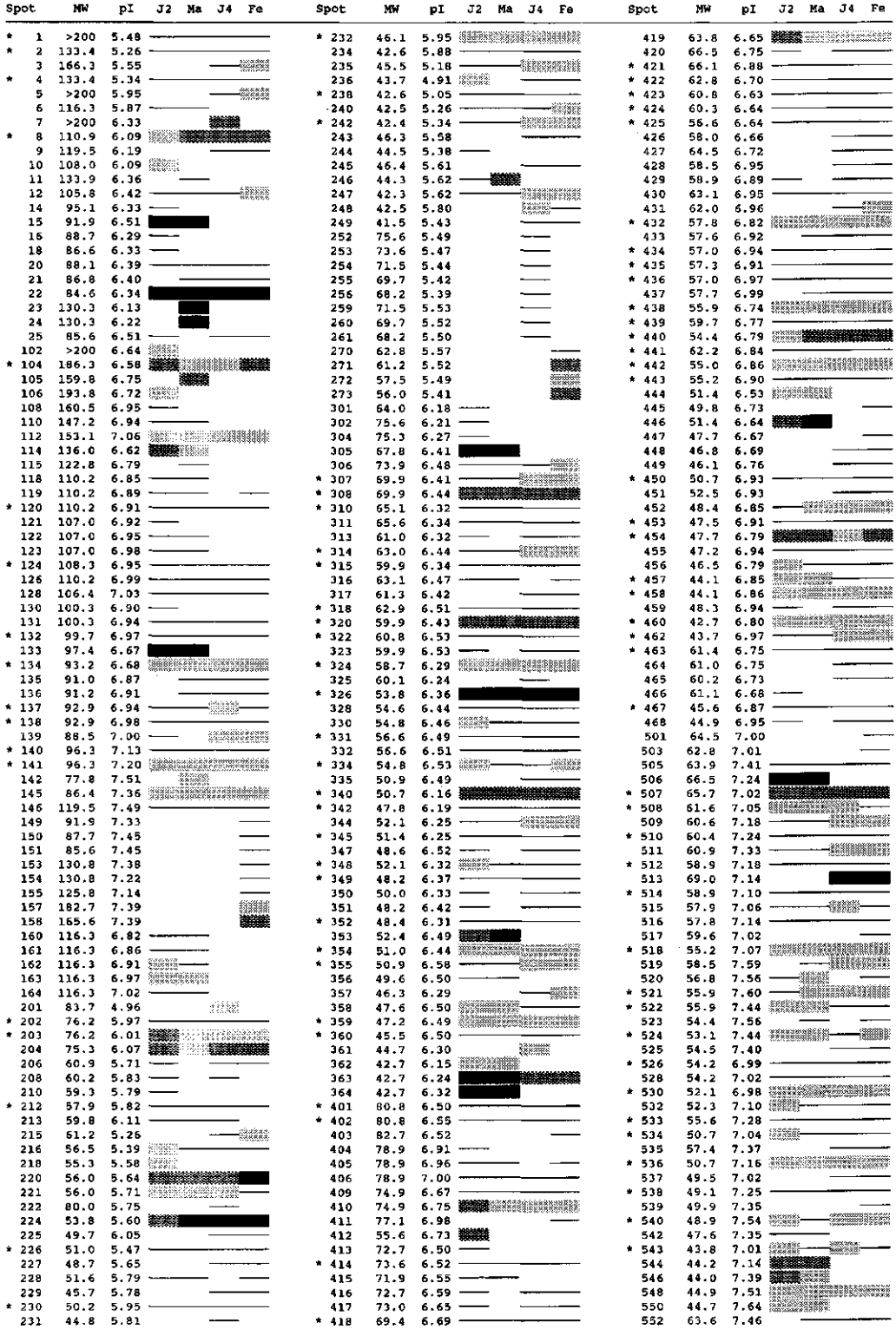
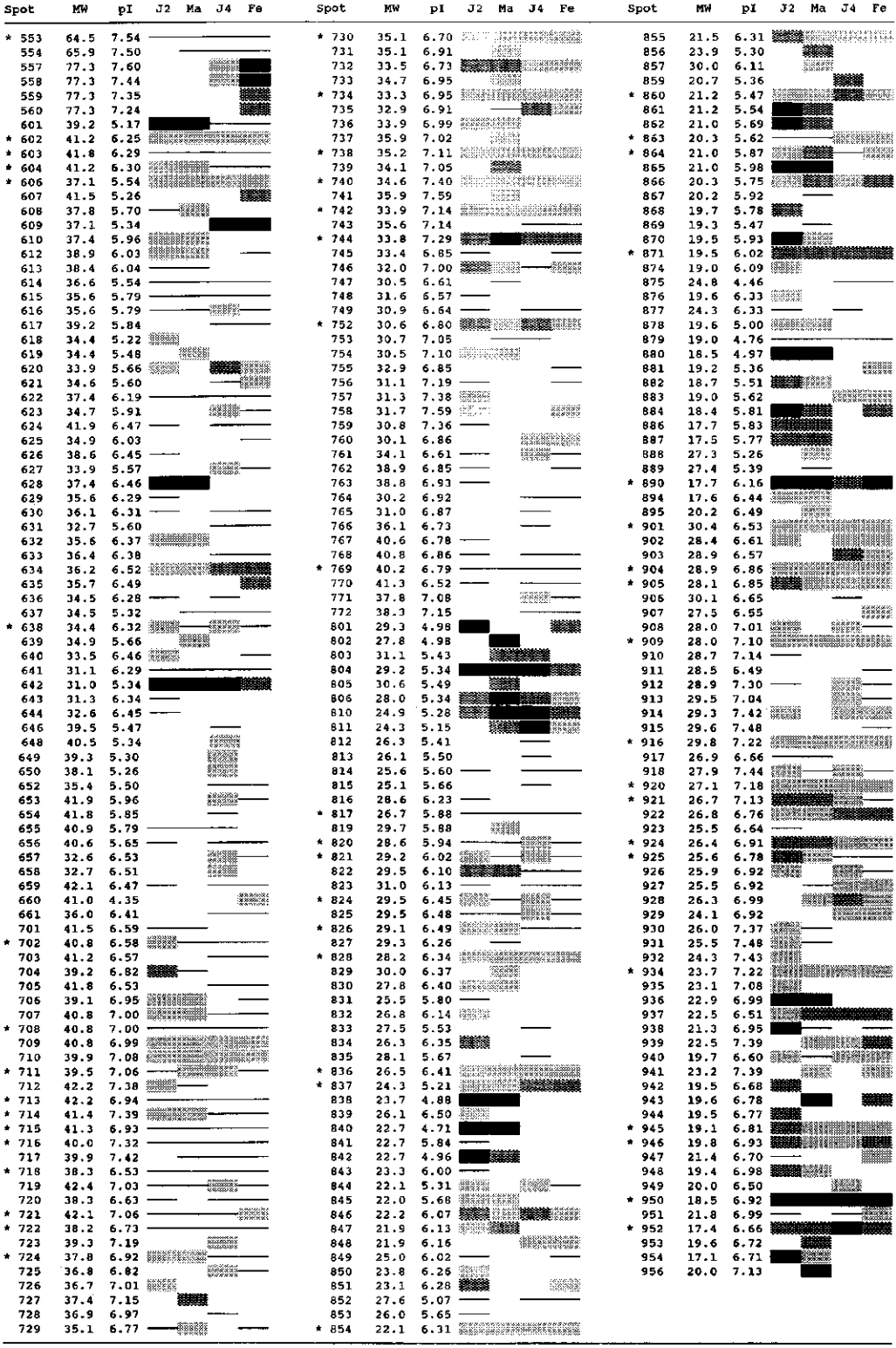


Fig 2.1. (For legend see page 23)


Spot MW pI J2 Ma J4 Fe
* 553 64.5 7.54
554 65.9 7.50
557 77.3 7.60
558 77.3 7.44
559 77.3 7.35
560 77.3 7.24
601 39.2 5.17
* 602 41.2 6.25
* 603 41.8 6.29
* 604 41.2 6.30
* 606 37.1 5.54
607 41.5 5.26
608 37.8 5.70
609 37.1 5.34
610 37.4 5.96
612 38.9 6.03
613 38.4 6.04
614 36.6 5.54
615 35.6 5.79
616 35.6 5.79
617 39.2 5.84
618 34.4 5.22
619 34.4 5.48
620 33.9 5.66
621 34.6 5.60
622 37.4 6.19
623 34.7 5.91
624 41.9 6.47
625 34.9 6.03
626 38.6 6.45
627 33.9 5.57
628 37.4 6.46
629 35.6 6.29
630 36.1 6.31
631 32.7 5.60
632 35.6 6.37
633 36.4 6.38
634 36.2 6.52
635 35.7 6.49
636 34.5 6.28
637 34.5 5.32
* 638 34.4 6.32
639 34.9 5.66
640 33.5 6.46
641 31.1 6.29
642 31.0 5.34
643 31.3 6.34
644 32.6 6.45
646 39.5 5.47
648 40.5 5.34
649 39.3 5.30
650 38.1 5.26
652 35.4 5.50
653 41.9 5.96
654 41.8 5.85
655 40.9 5.79
656 40.6 5.65
657 32.6 6.53
658 32.7 6.51
659 42.1 6.47
660 41.0 4.35
661 36.0 6.41
701 41.5 6.59
* 702 40.8 6.58
703 41.2 6.57
704 39.2 6.82
705 41.8 6.53
706 39.1 6.95
707 40.8 7.00
* 708 40.8 7.00
709 40.8 6.99
710 39.9 7.08
* 711 39.5 7.06
712 42.2 7.38
* 713 42.2 6.94
* 714 41.4 7.39
* 715 41.3 6.93
* 716 40.0 7.32
717 39.9 7.42
* 718 38.3 6.53
719 42.4 7.03
720 38.3 6.63
* 721 42.1 7.06
* 722 38.2 6.73
723 39.3 7.19
* 724 37.8 6.92
725 36.8 6.82
726 36.7 7.01
727 37.4 7.15
728 36.9 6.97
729 35.1 6.77
* 730 35.1 6.70
731 35.1 6.91
732 33.5 6.73
733 34.7 6.95
* 734 33.3 6.95
735 32.9 6.91
736 33.9 6.99
737 35.9 7.02
* 738 35.2 7.11
739 34.1 7.05
* 740 34.6 7.40
741 35.9 7.59
* 742 33.9 7.14
743 35.6 7.14
* 744 33.8 7.29
745 33.4 6.85
746 32.0 7.00
747 30.5 6.61
748 31.6 6.57
749 30.9 6.64
* 752 30.6 6.80
753 30.7 7.05
754 30.5 7.10
755 32.9 6.85
756 31.1 7.19
757 31.3 7.38
758 31.7 7.59
759 30.8 7.36
760 30.1 6.86
761 34.1 6.61
762 38.9 6.85
763 38.8 6.93
764 30.2 6.92
765 31.0 6.87
766 36.1 6.73
767 40.6 6.78
768 40.8 6.86
* 769 40.2 6.79
770 41.3 6.52
771 37.8 7.08
772 38.3 7.15
801 29.3 4.98
802 27.8 4.98
803 31.1 5.43
804 29.2 5.34
805 30.6 5.49
806 28.0 5.34
810 24.9 5.28
811 24.3 5.15
812 26.3 5.41
813 26.1 5.50
814 25.6 5.60
815 25.1 5.66
816 28.6 6.23
* 817 26.7 5.88
819 29.7 5.88
* 820 28.6 5.94
* 821 29.2 6.02
822 29.5 6.10
823 31.0 6.13
* 824 29.5 6.45
825 29.5 6.48
* 826 29.1 6.49
827 29.3 6.26
* 828 28.2 6.34
829 30.0 6.37
830 27.8 6.40
831 25.5 5.80
832 26.8 6.14
833 27.5 5.53
834 26.3 6.35
835 28.1 5.67
* 836 26.5 6.41
* 837 24.3 5.21
838 23.7 4.88
839 26.1 6.50
840 22.7 4.71
841 22.7 5.84
842 22.7 4.96
843 23.3 6.00
844 22.1 5.31
845 22.0 5.68
846 22.2 6.07
847 21.9 6.13
848 21.9 6.16
849 25.0 6.02
850 23.8 6.26
851 23.1 6.28
852 27.6 5.07
853 26.0 5.65
* 854 22.1 6.31
855 21.5 6.31
856 23.9 5.30
857 30.0 6.11
859 20.7 5.36
* 860 21.2 5.47
861 21.2 5.54
862 21.0 5.69
* 863 20.3 5.62
* 864 21.0 5.87
865 21.0 5.98
866 20.3 5.75
867 20.2 5.92
868 19.7 5.78
869 19.3 5.47
870 19.5 5.93
* 871 19.5 6.02
874 19.0 6.09
875 24.8 4.46
876 19.6 6.33
877 24.3 6.33
878 19.6 5.00
879 19.0 4.76
880 18.5 4.97
881 19.2 5.36
882 18.7 5.51
883 19.0 5.62
884 18.4 5.81
886 17.7 5.83
887 17.5 5.77
888 27.3 5.26
889 27.4 5.39
* 890 17.7 6.16
894 17.6 6.44
895 20.2 6.49
* 901 30.4 6.53
902 28.4 6.61
903 28.9 6.57
* 904 28.9 6.86
* 905 28.1 6.85
906 30.1 6.65
907 27.5 6.55
908 28.0 7.01
* 909 28.0 7.10
910 28.7 7.14
911 28.5 6.49
912 28.9 7.30
913 29.5 7.04
914 29.3 7.42
915 29.6 7.48
* 916 29.8 7.22
917 26.9 6.66
918 27.9 7.44
* 920 27.1 7.18
* 921 26.7 7.13
922 26.8 6.76
923 25.5 6.64
* 924 26.4 6.91
* 925 25.6 6.78
926 25.9 6.92
927 25.5 6.92
928 26.3 6.99
929 24.1 6.92
930 26.0 7.37
931 25.5 7.48
932 24.3 7.43
* 934 23.7 7.22
935 23.1 7.08
936 22.9 6.99
937 22.5 6.51
938 21.3 6.95
939 22.5 7.39
940 19.7 6.60
941 23.2 7.39
942 19.5 6.68
943 19.6 6.78
944 19.5 6.77
* 945 19.1 6.81
* 946 19.8 6.93
947 21.4 6.70
948 19.4 6.98
949 20.0 6.50
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* 952 17.4 6.66
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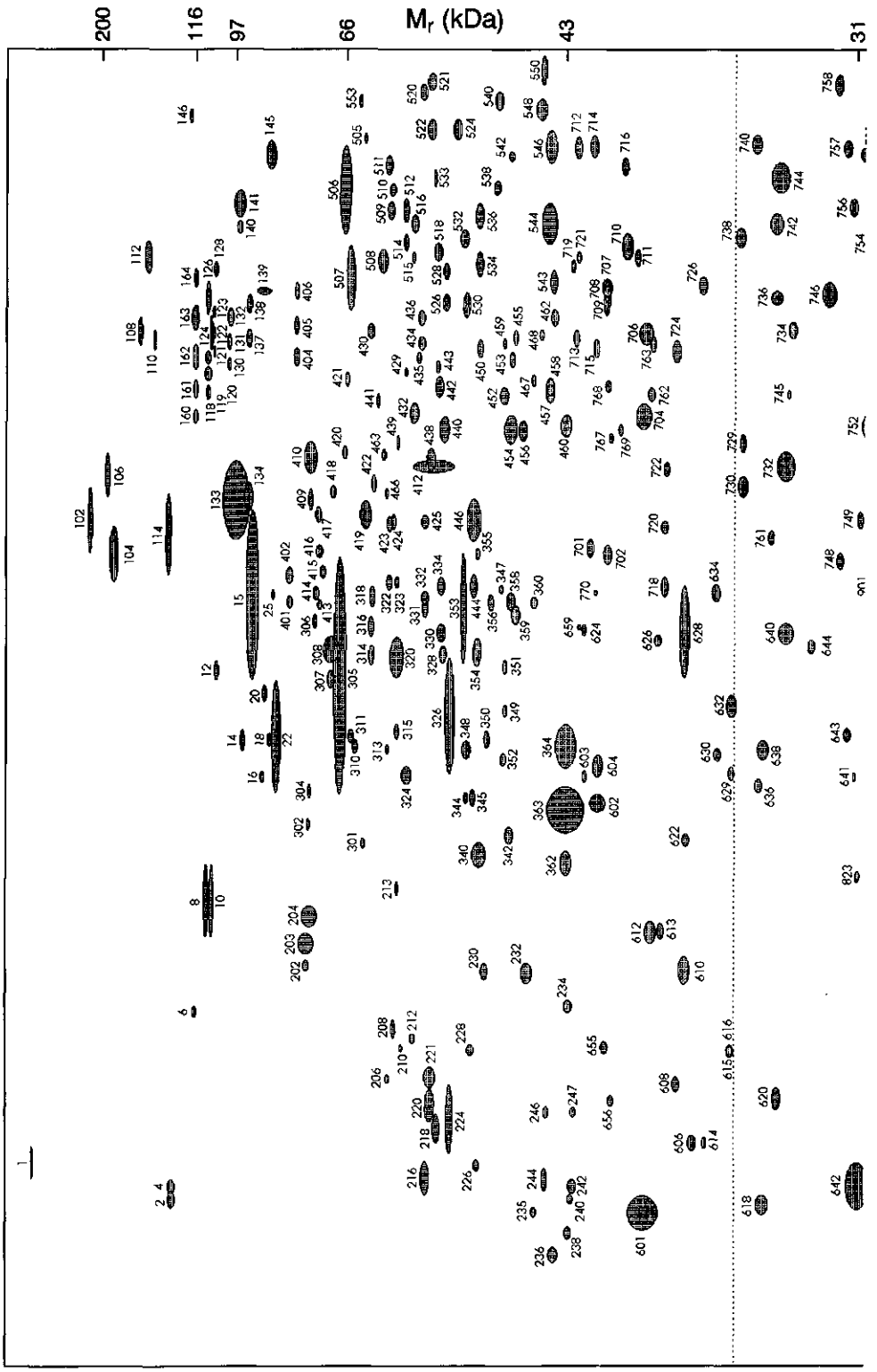
956 20.0 7.13


200
116
97
66
43
31
M_r (kDa)

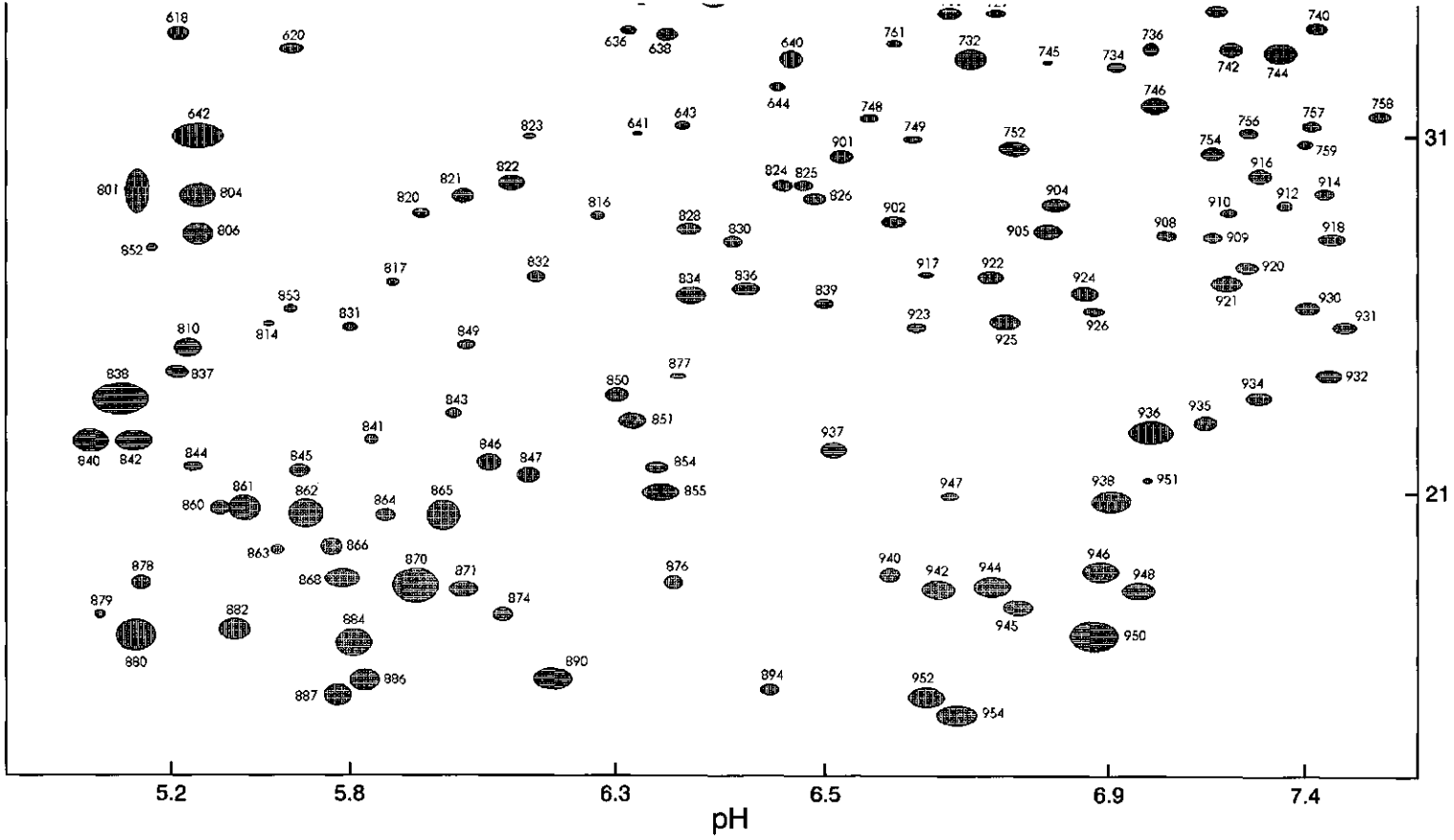
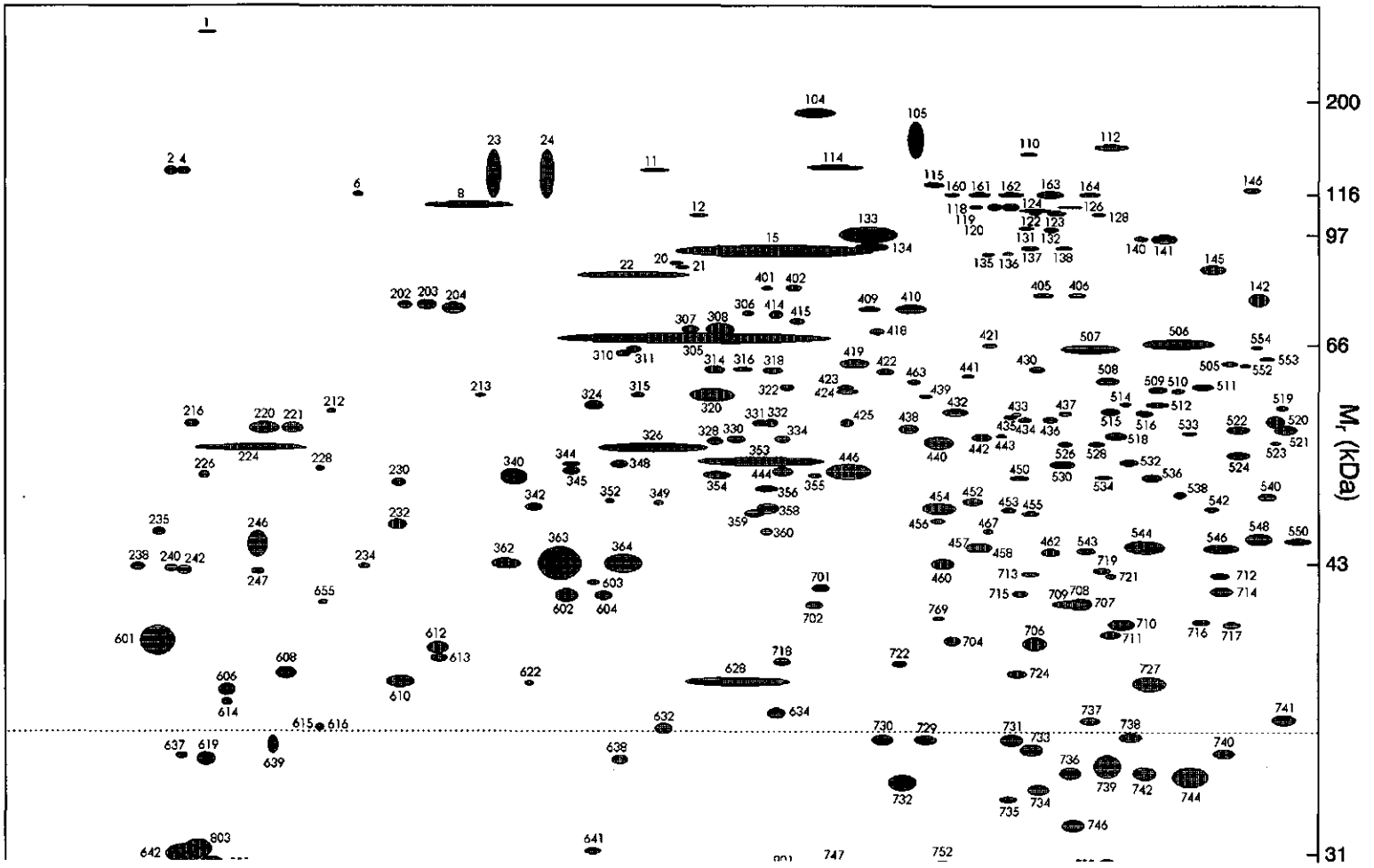


Fig. 2.2. Diagram of the two-dimensional protein pattern of 2nd-stage juveniles of *Globodera rostochiensis*


200
116
97
66
43
31
M_r (kDa)

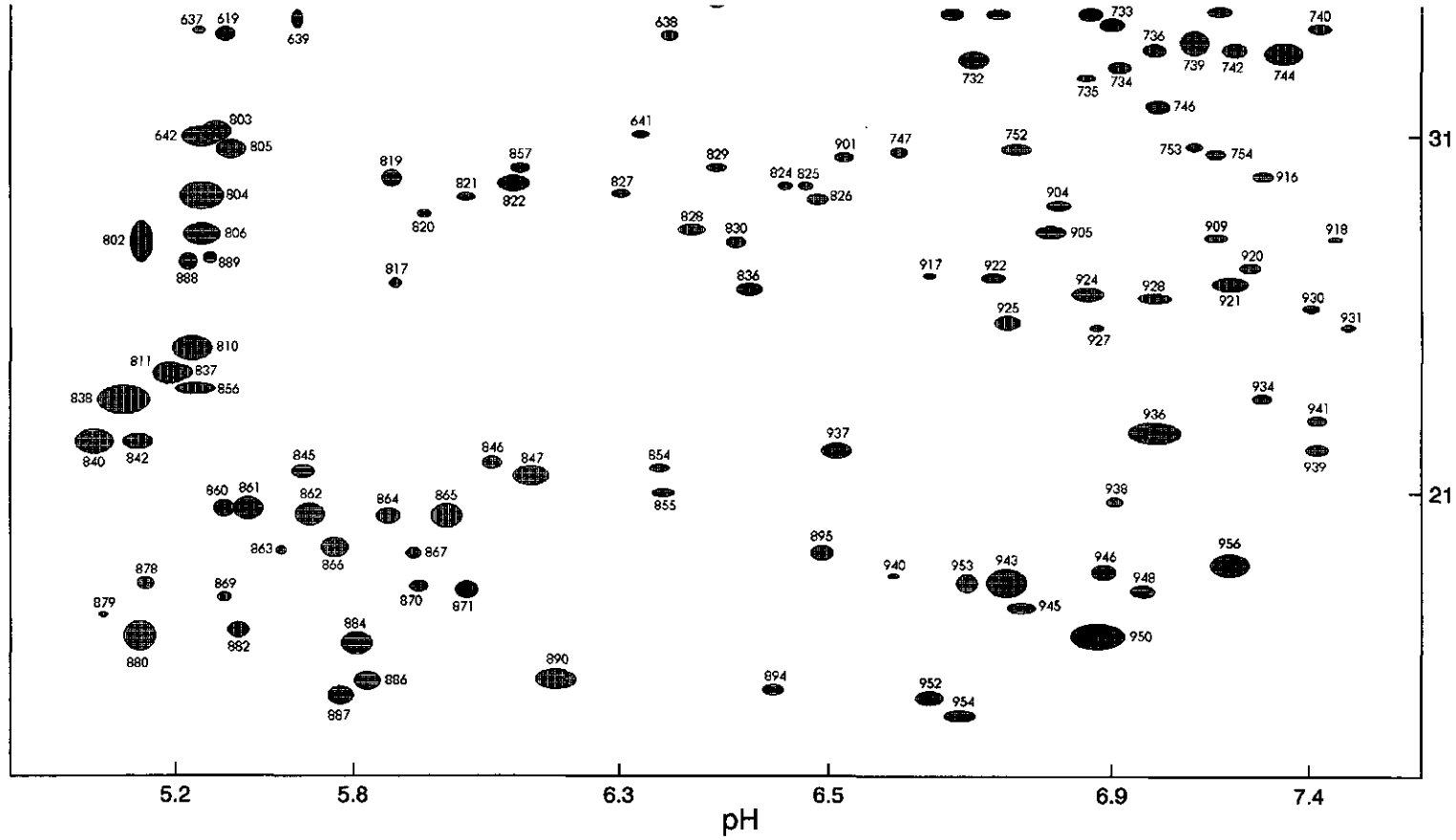
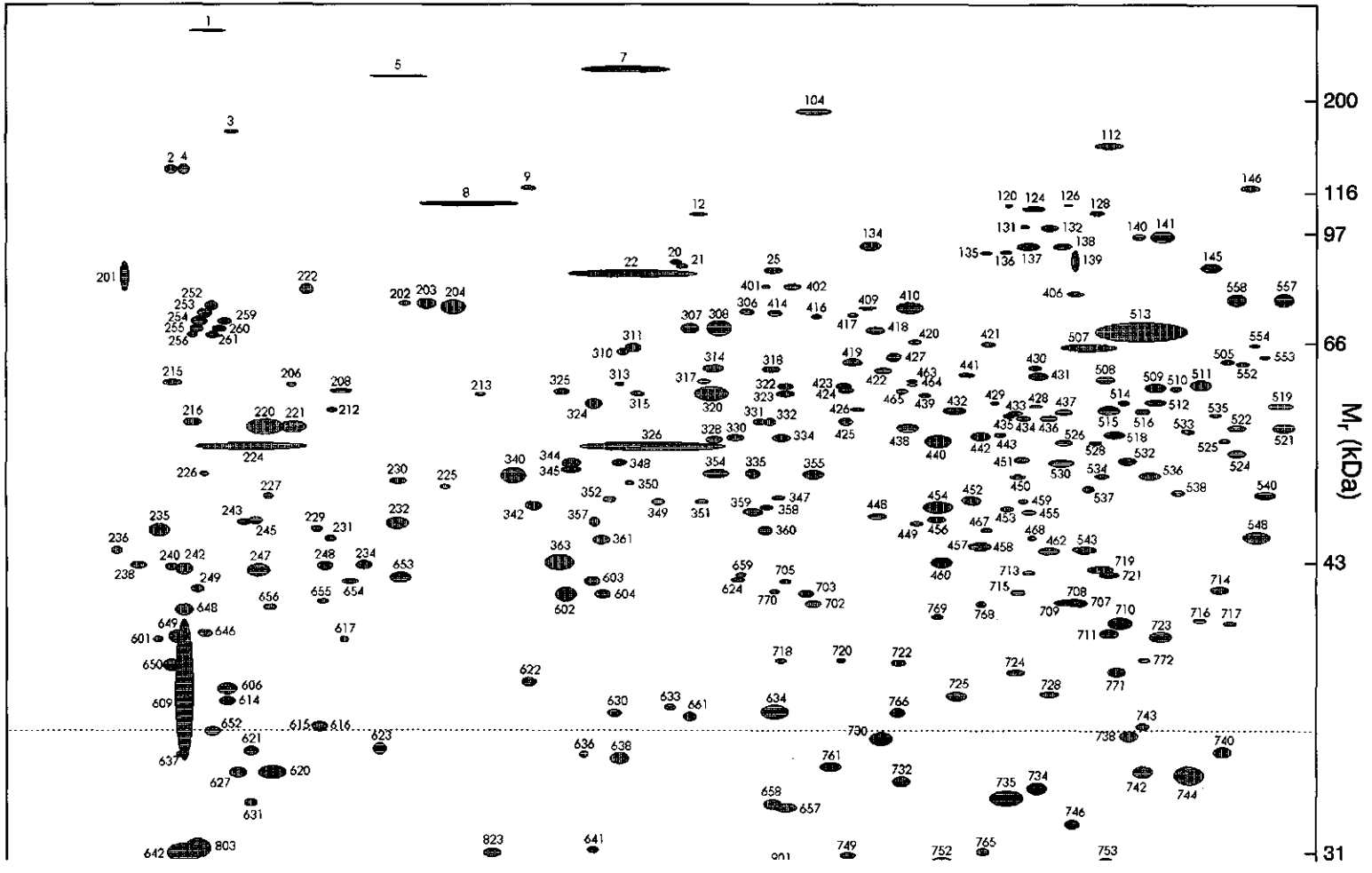


Fig. 2.3. Diagram of the two-dimensional protein pattern of adult males of *Globodera rostochiensis*


200
116
97
66
43
31
M_r (kDa)

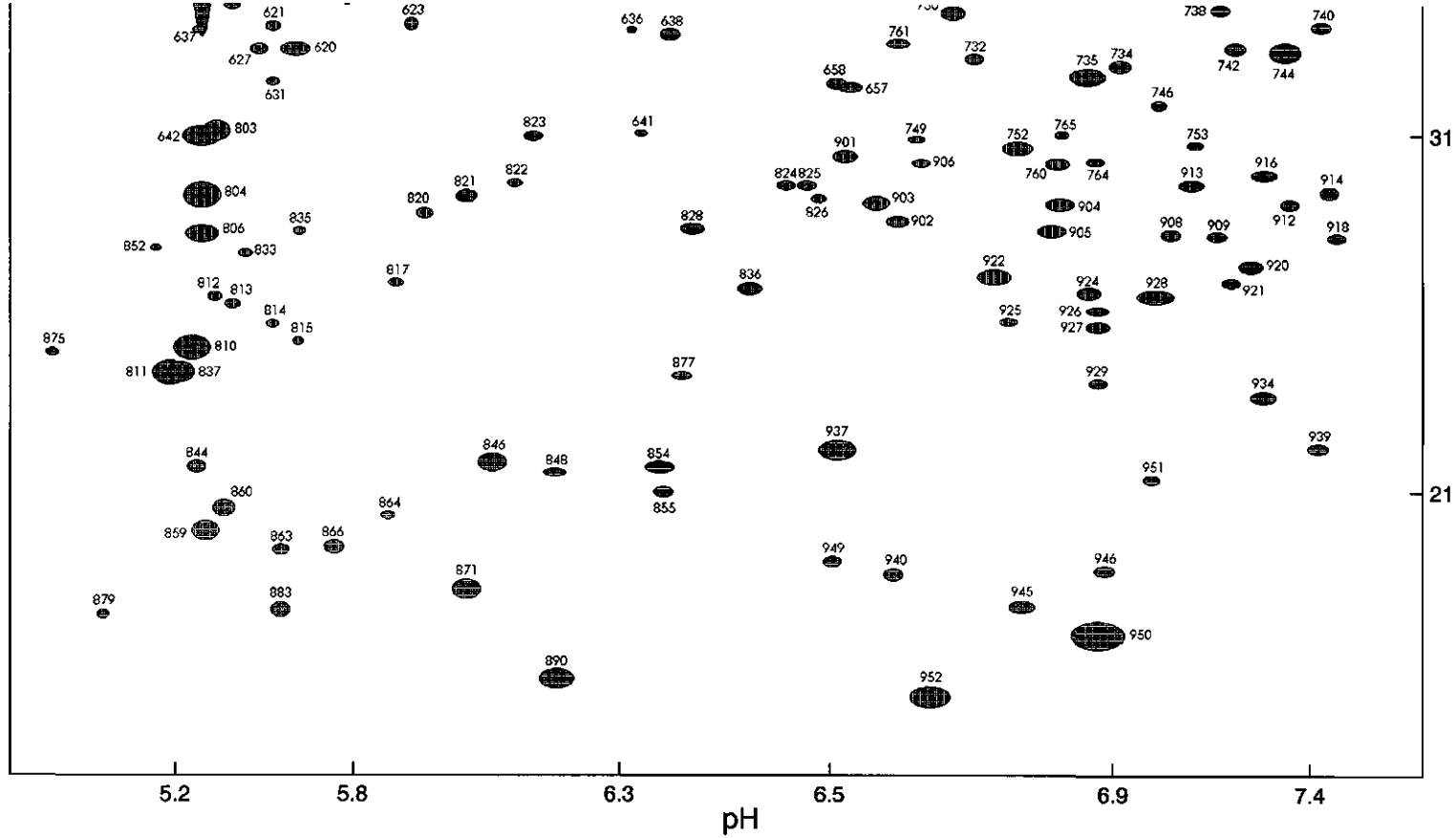
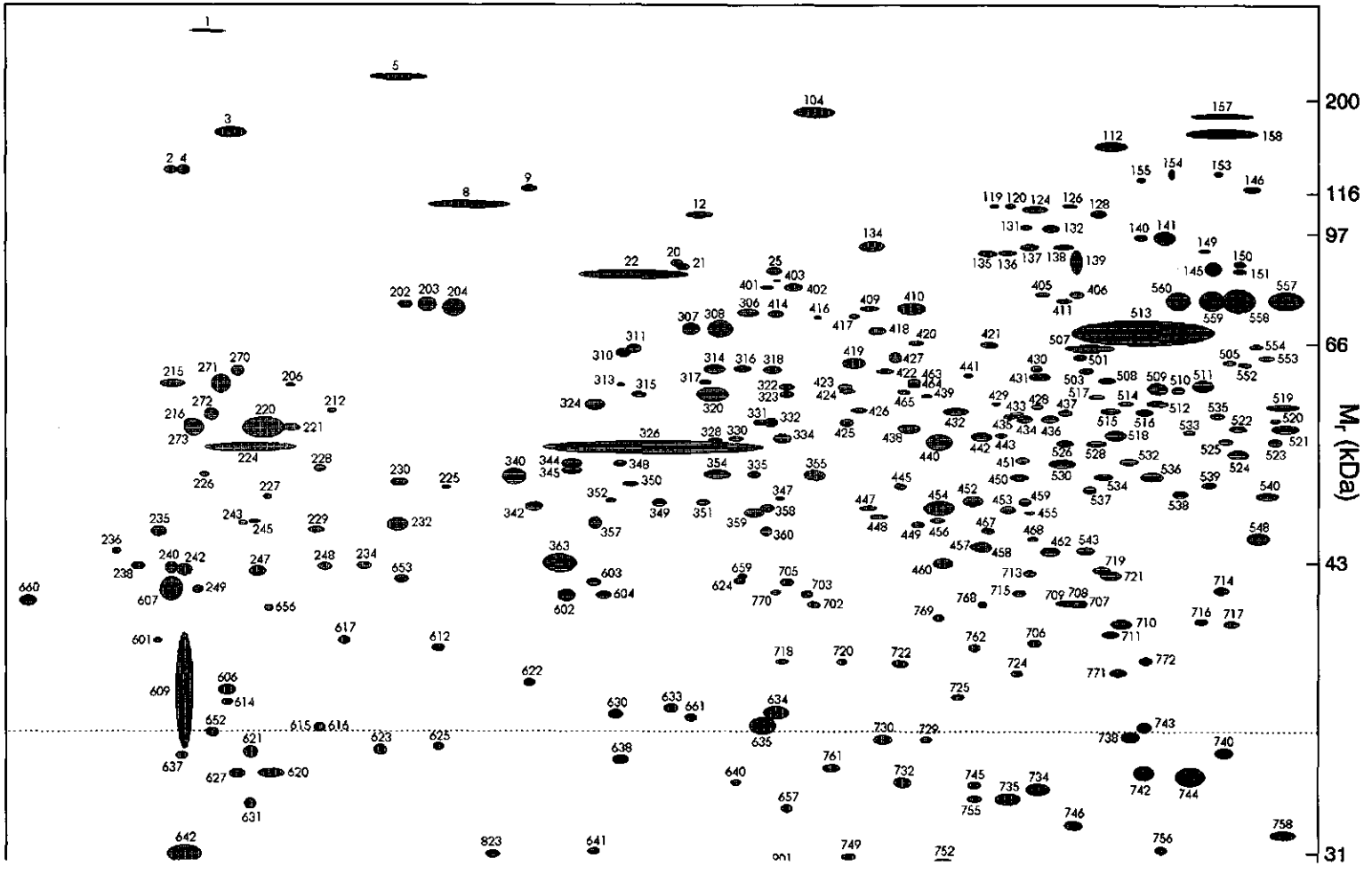


Fig. 2.4. Diagram of the two-dimensional protein pattern of 4th-stage female juveniles of *Globodera rostochiensis*


200
116
97
66
43
31
M_r (kDa)

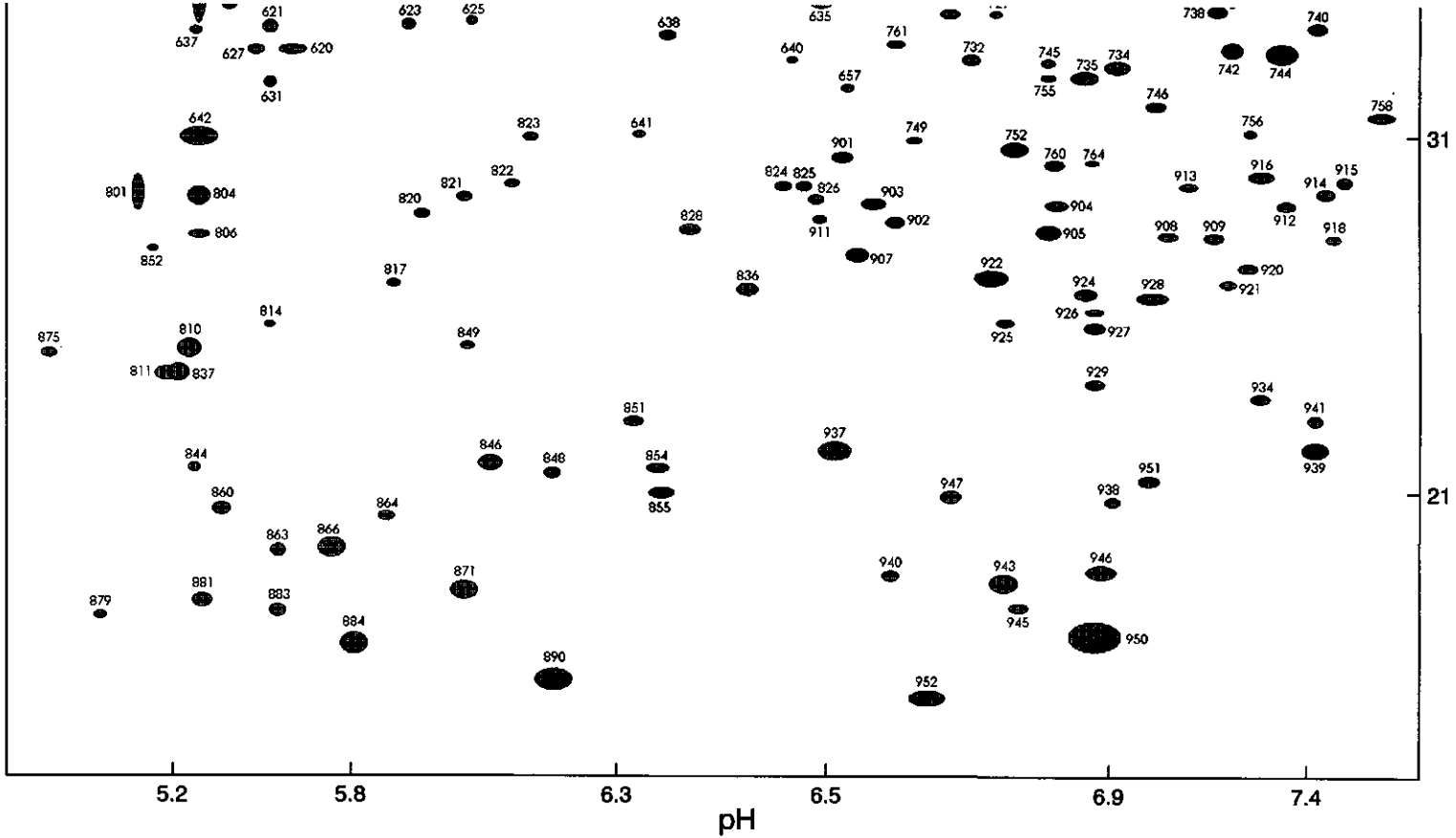


Fig. 2.5. Diagram of the two-dimensional protein pattern of adult females of *Globodera rostochiensis*

Table 2.2. Counts of protein spots unique to pairs of developmental stages of *Globodera rostochiensis*. (Above the diagonal all spots are shown; below the diagonal only spots of the size classes L and XL are included. J2, 2nd-stage juveniles; J4F, 4th-stage female juveniles).

	J2	Males	J4F	Females
J2		59	3	9
Males	19		2	3
J4F	0	1		54
Females	1	1	2	

Table 2.3. The number of proteins shared (below diagnonal) and similarity index values (above diagonal) between two-dimensional protein patterns of developmental stages of *Globodera rostochiensis*. (J2, 2nd-stage juveniles; J4F, 4th-stage female juveniles).

	J2	Males	J4F	Females
J2		0.674	0.535	0.584
Males	238		0.536	0.574
J4F	195	183		0.767
Females	217	200	276	

(text continued from page 23)

Numerical comparison between stages

Table 2.2 shows the numbers of spots that were unique to a given combination of stages. It was found that 59 spots were specifically present in J2 and males, and that 54 spots were solely visible in J4F and females. All other combinations of stages had far fewer pair-specific spots. This indicates that there is much similarity between the protein patterns of J2 and males, and of J4F and females. Remarkable in this respect is that in these two combinations the number of pair-specific spots exceeds the counts of corresponding stage-specific spots (Table 2.1). The similarity between J2 and males is further exemplified when examining pair-specific spots with a size of more than 5 mm^2 (Table 2.2). While in all other combinations few or no spots can be found that meet these criteria, J2 and males share 19 unique spots of the size classes L and XL.

The four stages were also compared by calculating similarity indices for the various combinations of protein patterns (Table 2.3). In determining the number of spots shared by a given pair of stages, a correction was used to account for variations in spot size. Only spots that did not differ significantly in size between the two stages compared were scored as common spots. Highest similarity indices were found for J4F and females (0.767) and for J2 and males (0.674), with decreased values (0.584 and less) for the other combinations.

Motile stages

Second-stage juveniles and males (Figs. 2.6 and 2.7) possess several large spots, that are either decreased in size or absent in J4F and females (Figs. 2.8 and 2.9). Characteristic in this respect are a number of proteins which presumably originate largely from the body-wall musculature, namely spot 133, spots 362-364, spot 601, and a streaked spot labelled 'MHC'. A detailed account of the identification of these proteins will be given in the discussion. In J4F and females spots 133, 362, 364, 601 and 'MHC' have all become either invisible or very small, and spot 363 is much reduced in size. Other major spots that disappear in the sedentary stages are labelled with small arrows in Figs. 2.6 and 2.7. (Because of its incomplete entry in the focusing gel the MHC-spot was not included in the numerical spot analysis, and it is therefore not shown in Fig. 2.1).

Sedentary stages

By contrast, few major spots can be found that are typical for both sedentary stages. Examples are spot 609, an acidic vertical streak extending from 34 to 41 kDa, and spot 513, which becomes very large in females. Furthermore, above spot 513, a train of spots is located (557-560), which is only partly visible in J4F, and becomes very abundant in females. In females, this spot train actually forms part of a large area filled with protein (labelled 'Y'), which extends beyond the acidic end of spot 513.

Stage-specific spots

The most conspicuous stage-specific proteins occurred in males (Fig. 2.7) where four unique extra large spots (23, 24, 802, and 956) were scored. Other prominent male-specific spots are 105, 142, 727, 739, and 953. Large spots specific for J2 (Fig. 2.6) are 412, 834, 942, and 944. In J4F (Fig. 2.8) only two stage-specific spots (7 and 859) were of a large size. Characteristic for this stage, however, are two diagonal rows of grey spots labelled 'T' (spot numbers 252-256, 259-261). Possibly, each of these spot trains represents a series of post-translational modifications of a single polypeptide. Typical spots for adult females (Fig. 2.9) are 271, 273, 157, 158, 607, and 635.

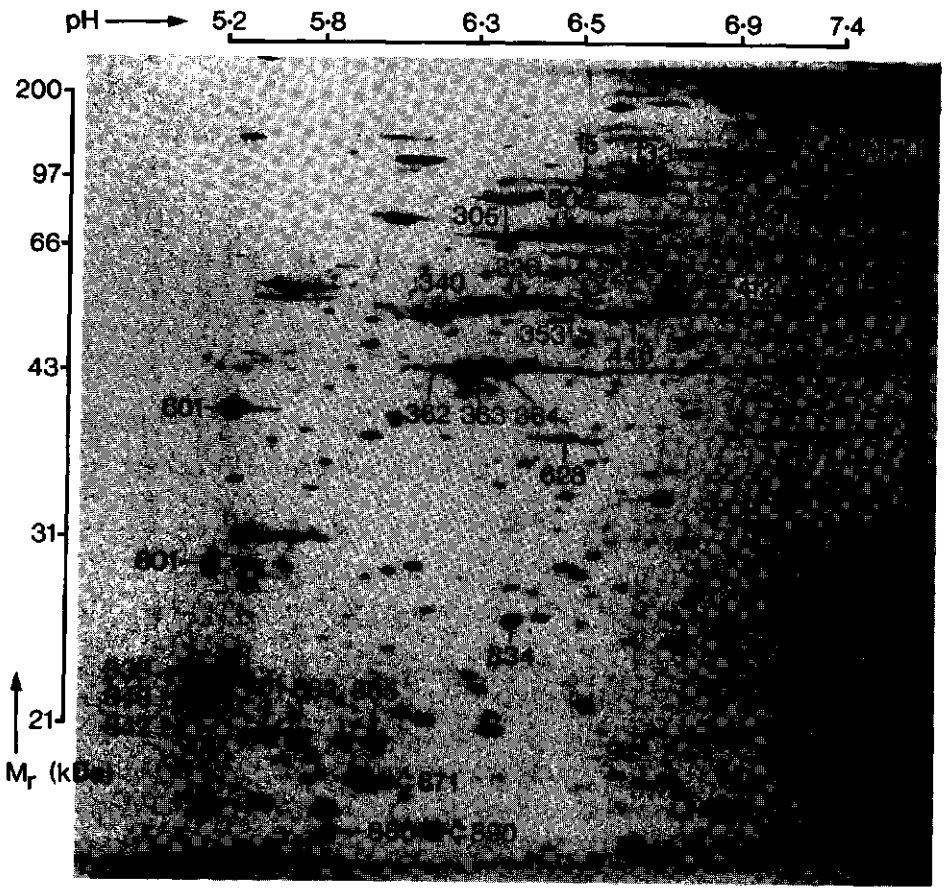


Fig. 2.6. Two-dimensonal protein pattern of 2nd-stage juveniles of *Globodera rostochiensis*. The spots marked with lines are referred to in the text. The small arrows indicate major spots occuring only in J2 and males. The open arrowheads indicate major invariant spots, which are present in all four stages.

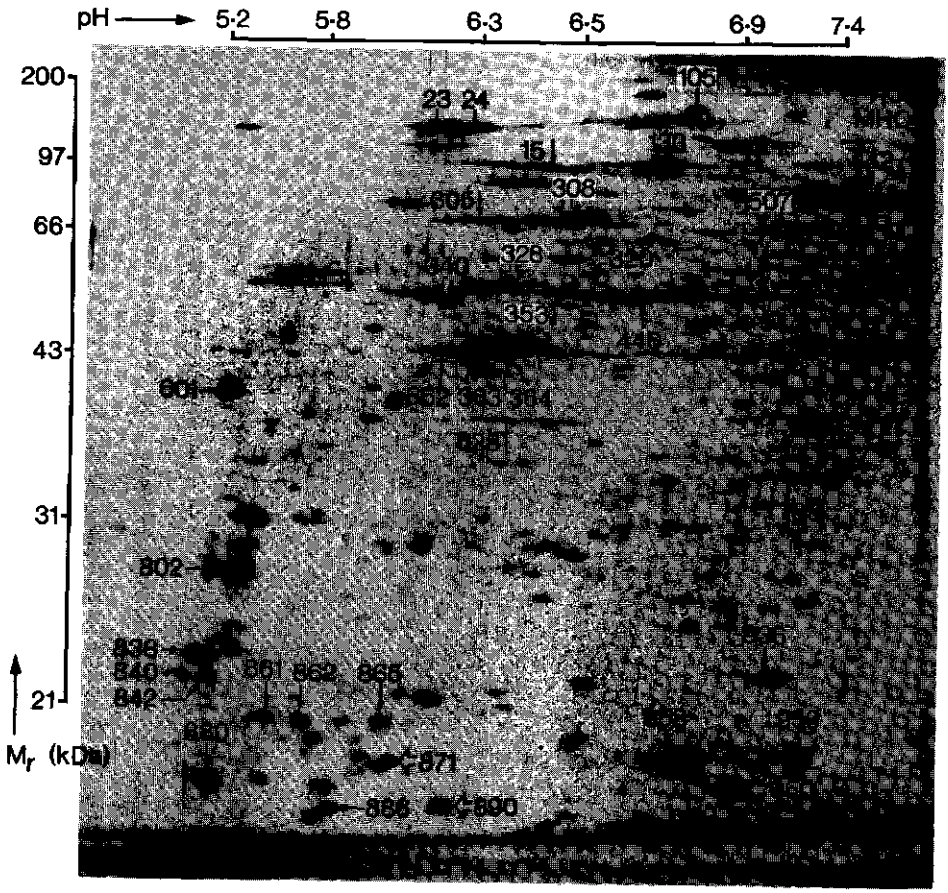


Fig. 2.7. Two-dimensonal protein pattern of adult males of *Globodera rostochiensis*. For markings see Fig. 2.6.

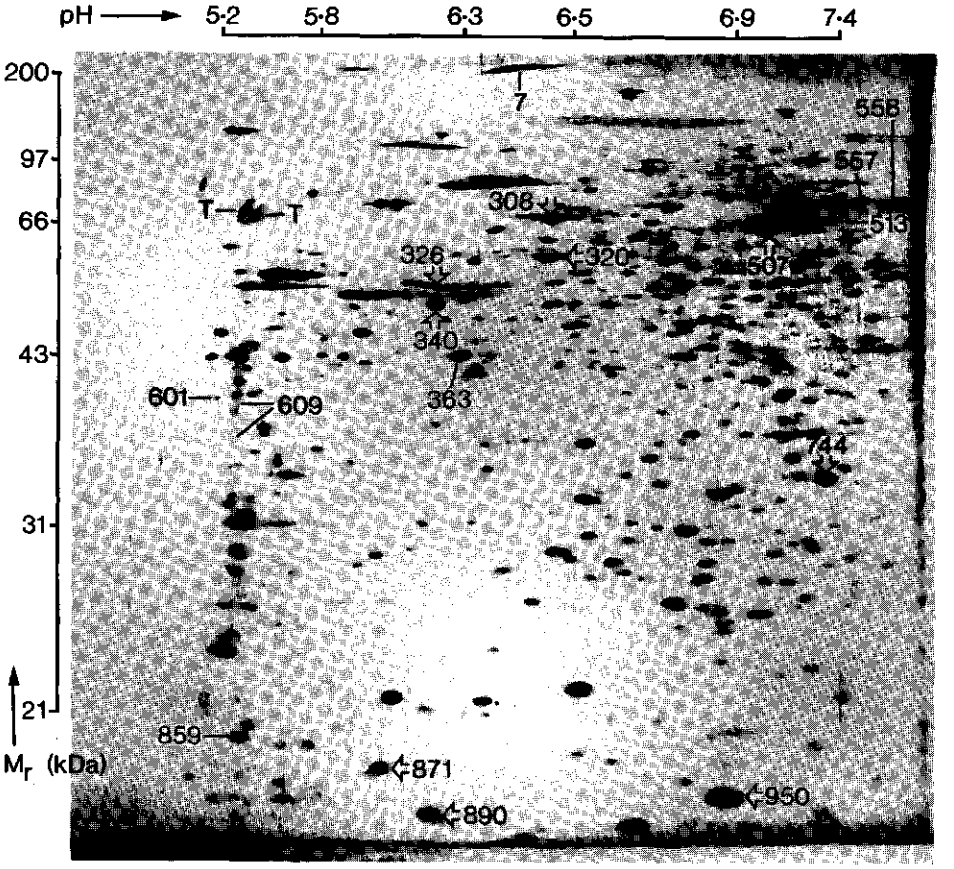


Fig. 2.8. Two-dimensonal protein pattern of 4th-stage female juveniles of *Globodera rostochiensis*. The spots marked with lines are referred to in the text. The open arrowheads indicate major invariant spots, which are present in all four stages.

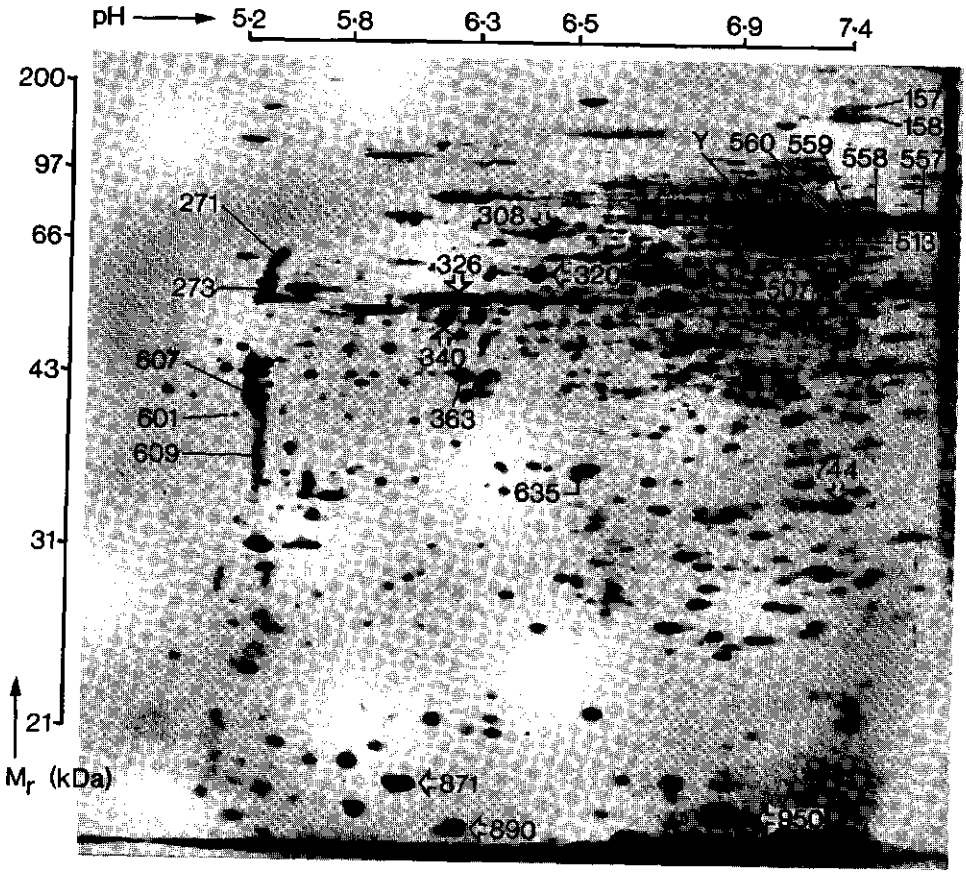


Fig. 2.9. Two-dimensonal protein pattern of adult females of *Globodera rostochiensis.* For markings see Fig. 2.8.

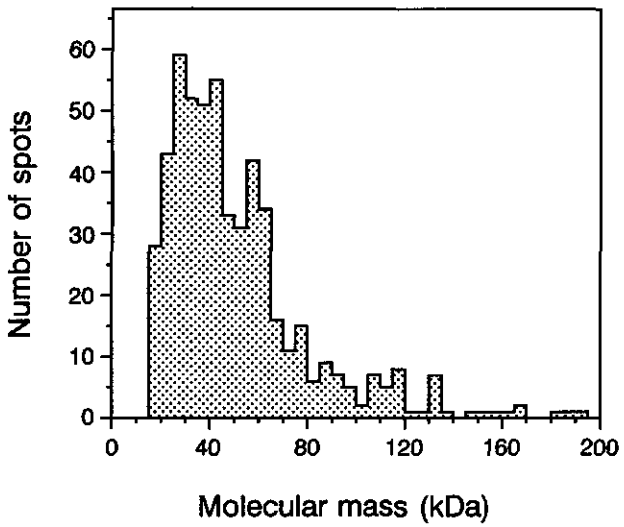


Fig. 2.10. Frequency distribution of apparent molecular weights of protein subunits in four developmental stages of *Globodera rostochiensis*. Proteins with a molecular mass of less than 17 kDa or more than 200 kDa are not included.

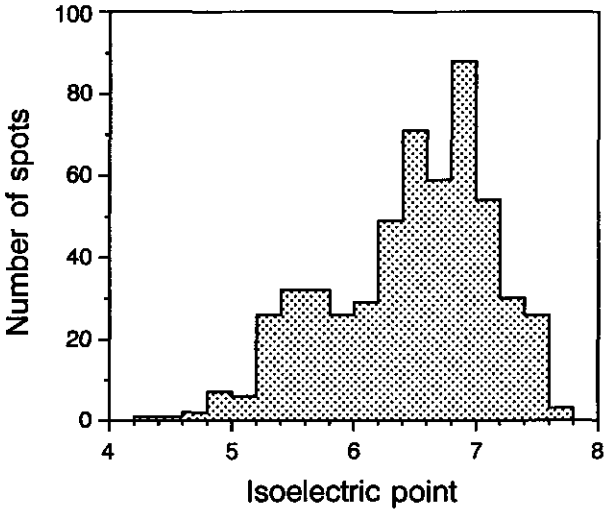


Fig. 2.11. Frequency distribution of estimated isoelectric points of proteins from four developmental stages of *Globodera rostochiensis*.

Molecular weights and isoelectric points

The distribution of the molecular weights of the protein spots tabulated in Fig. 2.1 is shown in Fig. 2.10. Treatment of protein samples with urea and sodium dodecyl sulfate causes dissociation of multimeric native proteins, and the distribution shown in Fig 2.10 therefore only depicts molecular weight values of protein subunits. The four spots listed in Fig. 2.1 with a molecular mass >200 kDa are omitted in Fig. 2.10. The distribution is skewed, with a maximum of about 55 spots/size class between 25 and 45 kDa. Then, the frequency drops to what seems to be second plateau of about 32 spots/size class between 45 and 65 kDa. Above 65 kDa there is another quick decline in the spot frequency. Only 23 polypeptides (4.3 %) had a size larger than 120 kDa. The average molecular mass was 51.0 kDa, with a standard deviation of 29.4 kDa (n=538). The Kolmogorov-Smirnov test indicated that the distribution of protein subunit molecular weights of *G. rostochiensis* can be described with a lognormal distribution. The distribution of the estimated isoelectric points is given in Fig. 2.11. A maximum in the spot frequency was found between pH 6.2 and pH 7.2.

Discussion

Using a sensitive silver stain, we distinguished 542 reproducible protein spots in two-dimensional protein patterns of 4 developmental stages of *G. rostochiensis*. No less than 401 of these spots varied in presence or size among the stages examined. This indicates that during post-embryonic development of the potato cyst-nematode at least 74% of the polypeptides are either modulated in their expression, removed by degradation, or subject to modifications. Johnson & Hirsh (1979) used 2D-PAGE to map the post-embryonic development of the free-living nematode *Caenorhabditis elegans*. By using a radioactive label, they could resolve 600-800 polypeptide spots in the 5 stages examined. Pulse labelling showed that 113 proteins (14-19%) were modulated in their synthesis, whereas continuous labelling revealed only 69 (9-12%) changing proteins. Since silver staining can be considered equivalent to continuous labelling methods in fluorography, this indicates that in *G. rostochiensis* the level of developmental protein variation is markedly increased compared to *C. elegans*. A high amount of protein variation was also found in the slime mold *Dictyostelium discoideum* (Cardelli *et al.*, 1985), in which 189 (47%) out of 400 spots changed their rate of synthesis during the developmental cycle. Since both *D. discoideum* and *G. rostochiensis* have a life cycle in which major changes in morphology and physiology occur, this explains the high percentage of changing proteins in these species.

Of the 210 spots that were consistently seen in all four stages, 141 (26%) did not show any significant variations in size throughout the stages examined. Many of these invariant proteins will therefore be organ-unspecific and involved in

elementary cell functions (Klose, 1982). Interesting in this respect is that in the soluble proteins of 3 mouse organs 23% of organ-unspecific proteins were found (Zeindl-Eberhart *et al.*, 1987), which is very similar to our percentage of invariant spots in *G. rostochiensis.*

High similarity indices were found for the two-dimensional protein patterns of J2 and males, and of J4F and females, with decreased values for the other pair-wise comparisons. These index values agree with the differences in anatomy and physiology between these stages: hatched juveniles and males are motile, vermiform, and non-feeding, whereas J4F and females are sedentary and parasitic. On the whole, the protein patterns of the sedentary stages were characterized by the absence of several major spots observed in the motile stages. This indicates that in *G. rostochiensis* the loss of motility is the major feature that differentiates the parasitic stages of from the non-parasitic ones.

In the electrophoresis patterns of J2 and males a number of abundant spots are found which we assume consist largely of body-wall muscle proteins. The relative positions of spots 133, 362-364, 601, and the streaked spot labelled 'MHC' correspond well with the positions of respectively paramyosin, actin, tropomyosin, and the myosin heavy chain subunit in the two-dimensional protein pattern of *C. elegans* muscle proteins (Epstein *et al.*, 1982). Also the apparent molecular weights of these spots are in agreement with published values for the corresponding muscle proteins in other nematodes (Harris & Epstein, 1977; Harris *et al.*, 1977; Schachat *et al.*, 1977; Kimura *et al.*, 1987; O'Donnell *et al.* 1989). Furthermore we found spot 601 to be heat stable (data not shown), which is a property of tropomyosins (Côté, 1983). Because of the incomplete focusing of the MHC spot, it was not possible to tell whether it may have contained multiple isoforms (Ardizzi & Epstein, 1987) or whether other high molecular weight proteins were located in this spot as well. In the sedentary female stages spots 133, 362-364, 601, and MHC disappear or are much reduced in size, and this corresponds well with the nearly complete disappearance of the body-wall musculature in these parasitic stages (Günther, 1972; Shepherd & Clark, 1978).

The spots labelled 362, 363, and 364 are presumably three isoelectric point variants of actin. Their occurrence is not uncommon, and it can be expected that these isoforms have different functions, or that they originate from different cell types or cell compartments (Garrels & Gibson, 1976; Tomlinson *et al.*, 1987). The absence of spots 362 and 364 in J4F and females suggests that these isoforms are restricted to the body-wall muscle cells. Spot 363 is the dominant isoform in hatched juveniles and males. In the two female stages it is reduced in size but still present as a fairly abundant spot. Because sedentary females possess very little muscle tissue, it is possible that spot 363 represents an actin isoform that occurs both in muscle cells and non-muscle cells. The quantity of spot 363 remaining visible in the sedentary females may therefore orginate largely from

cytoplasmic actin filaments (Bretscher, 1991).

In electrophoresis patterns of adult females a bulk amount of protein was located in spot 513, in the spot train 557-560, and in the protein accumulation labelled 'Y', which is situated above these spots. The abundance of these polypeptides and their occurrence in females makes it likely that some of them are yolk proteins. The free-living nematode *C. elegans* produces four yolk proteins, a doublet of 170 kDa, and two of 88 and 115 kDa respectively (Sharrock, 1984). Similarly, in the parasitic nematode *Heligmosomoides polygyrus* three polypeptides of 82 kDa, 115 kDa, and >140 kDa are believed to be yolk proteins (McGibbon & MacKinnon, 1990). Assuming a similar size distribution for the yolk proteins of *G. rostochiensis*, spots 557-560 with a molecular mass of 80 kDa, or the protein accumulation above them may well represent small yolk proteins.

Females of *G. rostochiensis* possess a cuticle that is much increased in thickness, and also different in structure, when compared with the cuticle of J2 and males (Wisse & Daems, 1968; Shepherd *et al.*, 1972). It can therefore not be excluded that some of the abundant proteins seen in J4F and females are from the cuticle wall, especially spot 513, which is already well represented in 4th-stage females.

In males the testis extends over about half their body length and a significant contribution of this gonad to the total protein pattern may be expected. Several of the major spots that were unique to males may therefore originate from the male reproductive organ. The highly conserved major sperm proteins of *Ascaris suum* and *C. elegans* have a molecular mass of about 15 kDa (Scott *et al.* 1989). Although polypeptides of this small size appear to fall just outside the range of our second dimension gels, it is tempting to speculate that one of the low molecular weight male specific spots, e.g. spot 953 or 956, represents a similar protein in *G. rostochiensis*.

The distribution of protein subunit molecular weights in *G. rostochiensis* was skewed and could be described with a lognormal distribution. Lognormal size distributions of total cell protein subunits have also been found in *Escherichia coli* and in mammalian cells (Savageau, 1986; Sommer & Cohen, 1980). Our average subunit size of 51.0 ± 29.4 kDa is very similar to the value of 50.4 ± 32.7 kDa obtained for 366 mammalian protein subunits (Sommer *et al.*, 1980). A periodicity in subunit molecular weights has been reported for proteins of *E. coli*, HeLa cells, and *Dictyostelium discoideum* (Savageau, 1986; Patton *et al.*, 1989). The peaks in our distribution around 35 and 55 kDa may indicate that size periodicity also exists in the polypeptides of *G. rostochiensis*. The relative absence in our analysis of spots above 120 kDa may not entirely reflect the natural situation, since it is possible that several high molecular weight polypeptides have failed to enter the focusing gel (Tanaka & Kawamura, 1988). Furthermore, silver staining may be less effective for high molecular weight proteins (Boxberg, 1988).

The estimation of pH-gradients and protein isoelectric points (pI) in 2D-electrophoresis is not accurate because of the high concentration of urea used in the isoelectric focusing gel. The presence of urea affects the pI of the separated proteins, the pI of the carrier ampholytes and also the pH-measurement itself (Gelsema *et al.*, 1979). By homogenizing focusing gel slices in water we tried to avoid some of these effects and measure reliable pH values for the reconstruction the gradient. However, the usefulness of this pH-gradient for estimating the pI of the separated proteins depends on the assumption that both the ampholytes and the proteins will show a similar shift in pI in response to urea (Gelsema *et al.*, 1978). Published data on the distribution of focusing pH values of proteins in 2D-electrophoresis patterns are still sparse. We found an asymmetrical distribution with a maximum around pH 6.8 for total protein extracts of *G. rostochiensis*, whereas *Dictyostelium* plasma membrane proteins showed a more symmetrical distribution with a maximum at pH 6.2 (Patton *et al.*, 1989).

In conclusion, two-dimensional electrophoresis of the potato cyst-nematode *G. rostochiensis* has revealed major differences in protein pattern between its two motile stages and two of its sedentary stages, confirming the differences in anatomy and physiology between these two developmental phases. Of particular interest is the resemblance in protein pattern between hatched juveniles and males. Since both are separated in time by intermediate parasitic forms, this implies that a substantial amount of protein recycling must occur during the development of juveniles to males. The numerical analysis of the electrophoresis patterns showed a high level of protein variation in *G. rostochiensis*. It is therefore concluded that the *G. rostochiensis* possesses a very dynamic protein metabolism, which can be fully ascribed to the specialized life cycle of this species. The results of our study emphasize the magnitude of the biochemical differences that can be encountered when studying parasite development with 2D-electrophoresis. The expression of proteins involved in the host-parasite interaction, such as secretory products or surface antigens, may well be restricted to a limited number of developmental stages, and a detailed knowledge of their occurrence throughout the life cycle may prove to be essential for successful parasite control.

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[1] Deceased August 1995.

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Chapter 3

Protein polymorphisms within *Globodera pallida* assessed with mini two-dimensional gel electrophoresis of single females

De Boer, J.M., Overmars, H.A., Bouwman-Smits, L., De Boevere, M., Gommers, F.J. & Bakker, J. 1992. Fundamental and Applied Nematology 15, 495-501.

Summary

Single females of *Globodera pallida* were analysed with mini two-dimensional gel electrophoresis (2-DGE) followed by a sensitive silver stain. Comparison of the protein patterns of 35 individuals from one population revealed 62 invariant protein spots and sixteen variant proteins having the typical characteristics expected from mutations that change the isoelectric point (IP-variants). The sixteen IP-variants are assumed to be encoded by sixteen alleles at eight putative loci. The frequencies of the presumed genotypes showed no significant deviations from Hardy-Weinberg equilibrium. The average heterozygosity per locus was 5 %. A detailed procedure for analysing minute amounts of protein with mini 2-DGE is described.

Key words: nematodes, potato, *Globodera*, pathotypes, virulence, allele frequencies, heterozygosity, electrophoresis

Introduction

In plant nematology the use of electrophoretic techniques for the discrimination of nematode populations has been largely confined to the analysis of mixtures of individuals. Standard electrophoretic techniques are too insensitive for analysing proteins from single nematodes of microscopic size. A number of investigators have developed micromethods of which micro disc electrophoresis (Dalmasso & Bergé, 1978; Bergé *et al.*, 1981) and more recently thin slab gel electrophoresis (Esbenshade & Triantaphyllou, 1985) have proved especially useful in studying the genetic variation between individuals. Various enzyme loci of *Meloidogyne* species have been screened for polymorphisms with these micromethods.

Another powerful technique for studying genetic variation is two-dimensional gel electrophoresis (2-DGE), according to O'Farrell (1975), which enables the examination of several hunderd polypeptides in a single sample. In a parallel paper, we reported on the genetic variation between populations of potato cyst-nematodes revealed by 2-DGE of mixtures of 100 individuals (Bakker *et al.*, 1992). The objective of the present study is to extend 2-DGE to the protein analysis of single females of potato cyst-nematodes and to examine the protein polymorphisms within a population of *Globodera pallida*. In addition we evaluated the accuracy of allele frequency data derived from mixtures of individuals (Bakker *et al.*, 1992) by comparing them with data obtained by electrophoresis of single individuals.

Materials and methods

Sample preparation

G. pallida population ROOK, classified as pathotype Pa_3, was obtained from the collection of the CPRO, Wageningen, the Netherlands. The population was originally collected at Valthe, the Netherlands. Adult, white females were reared on the susceptible cv. 'Eigenheimer' at 18 °C and 16 h daylength. Samples containing a mixture of individuals were prepared as described by Bakker & Bouwman-Smits (1988). Samples for electrophoresing single individuals were prepared by transferring one female into a homogenization tube (lenght 2 cm, inner diameter 1.7 mm). The tube was made from a glass capillary, which was heat sealed at one end. Single females were homogenized in 10 μl buffer with a small glass pestle, which was made by heating the tip of a capillary (outer diameter 0.9 mm). The size of the small ball formed at the end of the capillary was checked under the stereoscope to ensure a perfect fit inside the homogenization tube. The homogenization buffer was prepared by mixing 64.7 mg

urea, 57 µl 10 mM Tris-HCl, pH 7.4, and 13.3 µl of a solution containing 25 % 2-mercaptoethanol, 8 % (w/v) ampholytes pH 5-7 and 2 % (w/v) ampholytes pH 3-10. After homogenization, the extract was frozen for 30 min at -80 °C and after thawing immediately used for electrophoresis. Protein determinations were made according to Bradford (1976).

Mini two-dimensional electrophoresis

Chemicals for electrophoresis and Coomassie Brilliant Blue, G-250 were from Bio-Rad Laboratories (Hercules, USA). Silver nitrate and citric acid were from BDH Chemicals Ltd (Poole, England). All other chemicals were from Merck (Darmstadt, Germany). Distilled, deionized water was used for preparing the solutions and for the washing steps of the silver stain.

Mini two-dimensional electrophoresis was performed with the Mini Protean II 2-D Cell system of Bio-Rad Laboratories. Isoelectric focusing was performed in capillary tubes of 77 mm length and 1.1 mm inner diameter. The capillary tubes were cleaned by immersion in chromic acid and ethanol, and washed with distilled deionized water. The isoelectric focusing gels contained 3.78 % (w/v) acrylamide, 0.22 % (w/v) *bis*-acrylamide, 9.15 M urea, 1.6 % (w/v) ampholytes pH 5-7, 0.4 % (w/v) ampholytes pH 3-10, 0.013 % (w/v) ammonium persulfate, and 0.10 % (v/v) TEMED. The tubes were filled by capillary action with gel solution up to 10 mm below the top. They were pressed in a cushion of plasticine covered with parafilm, and allowed to polymerize for 1 h. Samples were applied on top of the gel with a glass capillary pipette. The remaining space in the focusing tubes was filled with sample overlay solution containing 5.2 mM Tris-HCl, pH 7.4, 8.41 M urea, 0.84 % (w/v) ampholytes pH 5-7, and 0.21 % (w/v) ampholytes pH 3-10. The cathode buffer was at the top of the focusing gels and contained 20 mM NaOH. The anode buffer was 10 mM phosphoric acid. Focusing was performed without pre-run and was accomplished with the following voltage schedule: 16.5 h 10 V, 90 min 180 V, 30 min 270 V, 80 min 578 V.

After focusing, the tube-gels were extruded in equilibration buffer containing 62.7 mM Tris-HCl, pH 6.8, 2.3 % (w/v) SDS and 10 % (v/v) glycerol. The separation gels were 84 × 59 × 0.75 mm (width × height × thickness). They contained 12.65 % (w/v) acrylamide, 0.35 % (w/v) *bis*-acrylamide, 375 mM Tris-HCl, pH 8.8, 0.1 % (w/v) SDS, and were polymerized with 0.05 % (w/v) ammonium persulfate and 0.05 % TEMED. The stacking gel (height 13 mm) was poured up to 1 mm below the top of the inner glass plate, and contained 3.894 % (w/w) acrylamide, 0.106 % (w/v) *bis*-acrylamide, 125 mM Tris-HCl, pH 6.8, 0.1 % (w/v) SDS, 0.05 % (w/v) ammonium persulfate, and 0.10 % (v/v)

TEMED. The focusing gels were positioned on top of the stacking gel by pushing them in the slit between the two glass plates. At both ends of the tube gel a few drops of SDS-sample buffer (86 mM Tris-HCl, pH 6.8, 2 % (w/v) SDS, 12.5 % (v/v) glycerol, 0.004 % Bromophenol Blue) were added. Both the upper and the lower buffer reservoirs were filled with a solution of 24.7 mM Tris, 192 mM glycine, and 0.1 % (w/v) SDS. Second dimension electrophoresis was performed with 10 mA/gel for 20 min, followed by 20 mA/gel for approximately 55 min, until the dye front had reached the bottom of the gel.

The gels were stained separately in plastic Petri dishes (diameter 13.5 cm) with 50 ml of solution for each step. First, the gels were transferred to 50 % (v/v) methanol, 10 % (v/v) acetic acid for 30 min. This was followed by incubation in 5 % (v/v) methanol, 7 % (v/v) acetic acid either for 30 min or overnight, and a fixation in 7 % (v/v) glutaraldehyde for 1 h. After four washing steps of 15 min in water, the gels were impregnated with ammoniacal silver solution (11.4 mM $AgNO_3$, 4.7 mM NaOH, 134 mM NH_3) for 20-25 min. After one washing step of 15 s and two 5 min washings in water, the gels were stained in 0.00925 % (w/v) paraformaldehyde, 0.0025 % (w/v) citric acid solution for 20-25 min. The staining reaction was stopped with water. Protein patterns were evaluated visually by superimposing the original gels on a bench viewer. Variant proteins spots (loci C, E, F, H, I, J) and a number of other conspicuous proteins which were also resolved with macro 2-DGE (Bakker *et al.*, 1992) were used as internal standards to estimate the pH gradient and molecular weights.

Results

To optimize the mini 2-DGE system, various conditions were tested by electrophoresing protein samples containing a mixture of individuals. A low initial voltage for isoelectric focusing proved to be essential for good quality protein patterns. The high initial voltages used in other studies (Poehling & Neuhoff, 1980; Neukirchen *et al.*, 1982; Sanderink *et al.*, 1988; Tanaka & Kawamura, 1988) resulted in streaking of the proteins, especially basic proteins with a high molecular weight. Application of a range of protein amounts demonstrated that 5 μg was the optimal quantity for mini 2-DGE. Approximately 350 protein spots were routinely visible with this amount (data not shown). Higher sample loads introduced streaking of the neutral and basic proteins and led to a loss of resolution in this area. Attempts to increase the sensitivity of the protein stain failed. Our staining procedure, which is essentially an adaptation of the ammoniacal silver stain of Oakley *et al.* (1980), was more sensitive than other staining methods (Oshawa & Ebata, 1983; De Moreno *et al.*, 1985;

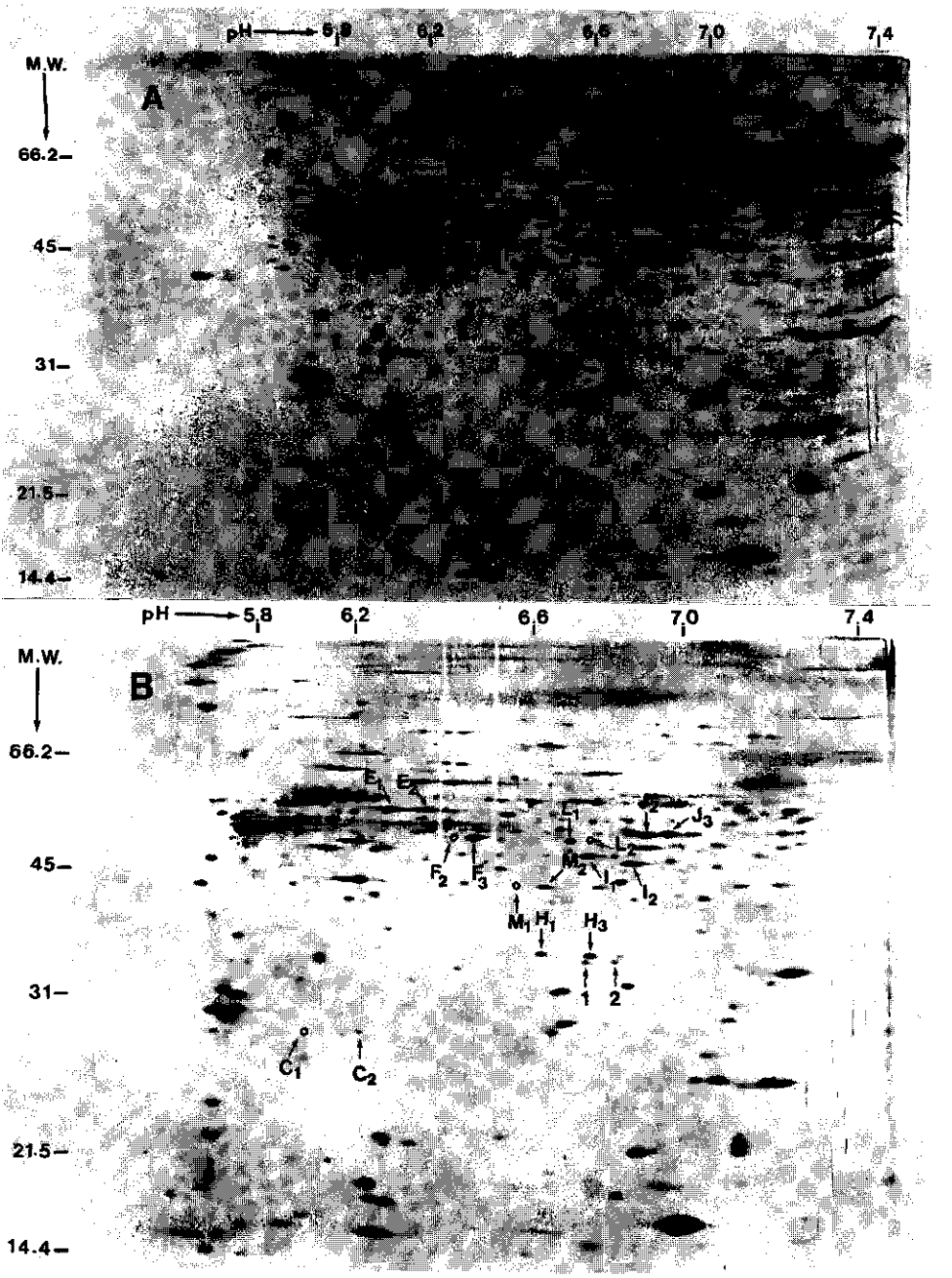
Chaudhuri & Green, 1987; Neuhoff *et al.*, 1988). Only the procedure of Poehling & Neuhoff (1981) was equally sensitive. The colour of the proteins visualized with our stain ranged from red, reddish brown, blackish brown, brownish grey to grey or any shade in between.

Thirty five individuals of *G. pallida* population ROOK were analysed with mini 2-DGE. Examples of the protein patterns of single females are shown in Fig. 3.1. The protein amount per female averaged 2.5 μg. The number of protein spots, including minor and faint spots, resolved per individual ranged from 233 to 439 with a mean of 360. Comparison of the protein patterns of 35 individuals revealed three types of protein spots: *i)* 62 invariant protein spots, which were consistently present in all 35 individuals, *ii)* 16 isoelectric point variants (IP-variants), which had the typical characteristics expected from mutations that change the isoelectric point (e.g. Fig. 3.2) and *iii)* a remaining group of more than 350 variant protein spots, which did not have the characteristics of the IP-variants. The latter group may also include genetically determined variant protein spots, but the majority of the variations is probably due to artefacts, such as, slight differences in the electrophoretic procedure, sample preparation and physiological state of the females.

The 16 IP-variants are assigned with a capital letter referring to the putative locus and an number referring to the allele. Corresponding IP-variants have moderately different isoelectric points, but have similar molecular weight, colour and quantity. As illustrated in Fig. 3.2, corresponding IP-variants are encoded by codominant alleles of which the products are synthesized in similar amounts. The genetic interpretation was confirmed by a chi-square test showing that the observed number of genotypes did not deviate significantly from the number expected under Hardy-Weinberg equilibrium conditions (Table 3.1).

The average heterozygosity per locus was 5 % and was calculated according to Leigh Brown & Langley (1979) by assuming that the 62 invariant spots and 16 IP-variants represent the protein products of 70 loci. Obviously this is a coarse estimate. As has been established in other studies (Racine & Langley, 1980) some alleles will display more than one protein spot; we also noticed this phenomenon. Each allele of locus H displays probably two protein spots (Fig. 3.1). The presence (or absence) of allele H_1 was accompanied in each individual by the presence (or absence) of protein spot no. 1 (Fig. 3.1). Similar results were obtained for allele H_3 and protein spot no. 2 (Fig. 3.1). The protein spots no. 1 and no. 2 were therefore not recorded as separate IP-variants.

The IP-variant loci C, E, F, H, I, J (Fig. 3.1) were also recorded as polymorphic for population ROOK in a previous study with macro 2-DGE (Bakker *et al.*, 1992). As shown in Table 3.2 the allele frequency data obtained by mini


pH → 5.8
6.2
6.6
7.0
7.4
M.W.
66.2
45
31
21.5
14.4
A
B
E1
E2
F1
F2
F3
J3
L2
M2
I1
I2
M1
H1
H3
1
2
C1
C2

2-DGE of individuals are within the same range as the data obtained by macro 2-DGE of a mixture of 100 individuals. In the latter case the relative protein quantities of the variant protein spots were used as a measure for the allele frequencies (Bakker *et al.*, 1992). The largest differences between the two methods were observed for locus E and I. The macro 2-DGE data for these two loci fall just outside the 95 % confidence interval of the mini 2-DGE data (Table 3.2).

Differences in experimental conditions and data collection mean that not all the protein polymorphisms detected with macro 2-DGE are revealed with mini 2-DGE and *vice versa*. Allele F_1 having a frequency of 0.03 (Bakker *et al.*, 1991) was probably not traced in this study because of the smaller number of individuals sampled (35 *vs* 100). The IP-variant loci A, D and K (Bakker *et al.*, 1992) were not detected with mini 2-DGE and the IP-variant loci L and M (Fig. 3.1) were not resolved with macro 2-DGE.

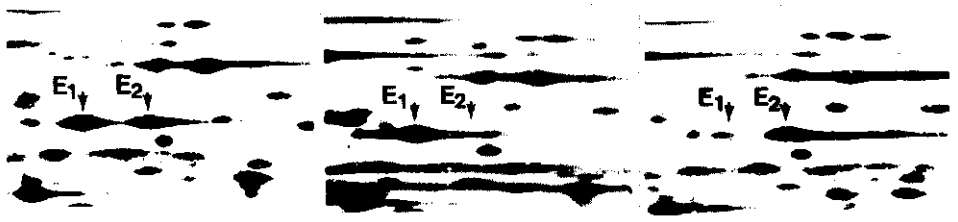


Fig. 3.2. Details of protein patterns made by electrophoresing single individuals of *Globodera pallida* population ROOK. The positions of the isoelectric point varants (IP-variants) are designated with capitals and numbers referring to the putative loci and alleles, respectively. The presumed genotypes of the three individuals are: E_1E_2 (left), E_1E_1 (middle), E_2E_2 (right).

Fig. 3.1 (on opposite page). Protein patterns of single individuals from *Globodera pallida* population ROOK. The positions of the isoelectric point variants (IP-variants) detected by the comparison of 35 individuals are shown. The capitals and numbers refer to the putative loci and alleles, respectively. The presumed genotypes for the individual shown in **A** are: C_1C_1, E_1E_2, F_2F_3, H_3H_3, I_1I_2, J_2J_2, L_1L_1 and M_2M_2. The presumed genotypes for the individual shown in **B** are: C_2C_2, E_1E_2, F_3F_3, H_1H_3, I_1I_2, J_2J_3, L_1L_1, M_2M_2. The proteins indicated with arabic numbers (no. 1 and no. 2) are referred to in the text. Molecular masses are given in kilodaltons.

Table 3.1. Hardy-Weinberg Equilibrium Test on presumed genotypes (1) detected in *G. pallida* population ROOK after mini 2-DGE of 35 individuals

	C_1C_1	C_1C_2	C_2C_2	E_1E_1	E_1E_2	E_2E_2	F_2F_2	F_2F_3	F_3F_3	H_1H_1	H_1H_3	H_3H_3	I_1I_1	I_1I_2	I_2I_2	J_2J_2	J_2J_3	J_3J_3	L_1L_1	L_1L_2	L_2L_2	M_1M_1	M_1M_2	M_2M_2
Observed	8	14	13	4	17	14	6	15	14	5	18	12	6	18	11	14	17	4	23	12	0	4	12	19
Expected	6.4	17.1	11.4	4.5	16.0	14.4	5.2	16.6	13.2	5.6	16.8	12.6	6.4	17.1	11.4	14.4	16.0	4.5	24	9.9	1	2.8	14.3	17.8
Total χ^2(2)	1.17			0.014			0.325			0.177			0.087			0.116			0.53			0.388		

(1) The presumptive loci and alleles of the isoelectric point variants (IP-variants) are designated with capitals and arabic numbers, respectively.
(2) One degree of freedom $\chi^2 = 3.84$ corresponds with the 5% level of significance. Yate's correction was applied in case the expected number of one genotype was smaller than five (Ayala & Kriger, 1980).

Table 3.2. Allele frequencies of isoelectric point variants (IP-variants) (1) estimated by mini 2-DGE of single individuals and macro 2-DGE of a mixture of individuals (2).

	C_1	C_2	E_1	E_2	F_1	F_2	F_3	H_1	H_3	I_1	I_2	J_2	J_3	L_1	L_2	M_1	M_2
mini 2-DGE	0.43	0.57	0.36	0.64	0	0.38	0.62	0.40	0.60	0.43	0.57	0.64	0.36	0.83	0.17	0.29	0.71
macro 2-DGE (2)	0.35	0.65	0.23	0.77	0.03	0.29	0.68	0.42	0.58	0.30	0.70	0.59	0.41	n.d.	n.d.	n.d.	n.d.
95 % interval (3)	0.31-0.55		0.25-0.47			0.26-0.50		0.28-0.52		0.31-0.55		0.53-0.75		0.74-0.92		0.18-0.40	
pI (4)	5.96	6.20	6.23	6.29	6.32	6.42	6.48	6.62	6.78	6.71	6.84	6.90	6.97	6.69	6.77	6.56	6.63
MW (5)	28		60		54			36		50	49	56		53		42	
colour (6)	r.		r.b.		r.b.			b.b.		r.b.		r.b.		r.b.		r.b.	

(1) The presumptive loci and alleles of the IP-variants are designated with capitals and arabic numbers, respectively.
(2) Data from Bakker *et al.*, 1992.
(3) Confidence intervals of the allele frequencies estimated by electrophoresing single individuals. Only the range of the first allele is given.
(4) Isoelectric points.
(5) Molecular masses in kilodaltons.
(6) Red (r.), reddish brown (r.b.) and blackish brown (b.b.).

Discussion

Since its introduction 2-DGE has become a wide-spread technique in various disciplines. As an alternative to radioactive labelling methods and Coomassie Blue staining, ultra sensitive silver stains were developed. With the introduction of micro 2-DGE (Poehling & Neuhoff, 1980) the sensitivity of protein detection was further increased. However, the use of micro 2-DGE is still limited and applications deal mainly with the analysis of a low number of polypeptides (Poehling *et al.,* 1980; Ohms & Heinicke, 1985; Manabe *et al.,* 1987; Sanderink *et al.*, 1988). A new development in 2-DGE is the use of mini gels (Sakurai *et al.*, 1986; Tanaka & Kawamura, 1988), which have a size intermediate between conventional and micro gels. As shown in this report mini 2-DGE allows the analysis of complex protein samples containing only a few μg of total protein. The high resolution of conventional 2-DGE is retained and the sensitivity of detection is increased by about a factor ten. In addition the system is easier to handle, faster, and very economical in the use of chemicals.

Analysing and evaluating 100 or more proteins on a single 2-DGE pattern is evidently more difficult and less straightforward than interpreting isozyme patterns. In this report we used stringent criteria to collect data from 2-DGE patterns. Only protein variations having the typical characteristics of corresponding allele products differing in one or a few net charge-changing amino acids were included. The assumption that such variations are indeed encoded by alleles at a single locus is supported by the observation that the relative frequencies of the homozygous and heterozygous genotypes do not deviate from Hardy-Weinberg expectations. Other types of variation are difficult to assess by electrophoresis of single individuals. During the course of this study we observed numerous qualitative and quantitative differences for which no homologues could be detected. These variations are difficult to evaluate, because genetically determined variants cannot be distinguished from artefacts due to, for example, experimental variations or differences in physiological or developmental stage.

Genetic variation in abundant proteins revealed by 2-DGE has been studied in organisms such as *Drosophila melanogaster* (Leigh Brown & Langley, 1979), mice (Racine & Langley, 1980) and man (McConkey *et al.*, 1979; Rosenblum *et al.*, 1983). In these studies the average heterozygosity per locus based on variations in net charge ranged from 1 to 6 %. The variability of *G. pallida* population ROOK is not exceptional (5 %). The actual heterozygosity is evidently much higher. Only about 30 % of the amino acid substitutions result in a displacement in the isoelectric focusing dimension and other types of mutations resulting in qualitative or quantitative variations are not recorded. Quantitative

and qualitative variations, for example due to mutations in regulatory genes, seem a prominent group among the variants resolved by 2-DGE. Klose (1982) studied inbred strains of mice and demonstrated that only 13 % of the protein variants resulted from changes in net charge and that the vast majority (87 %) of the variants were expressed as quantitative or qualitative differences.

In a parallel report we estimated the genetic relationships between potato cyst-nematode populations by electrophoresing mixtures of individuals and using the ratio between the potein quantities of corresponding IP-variants as a measure for the allele frequencies (Bakker *et al.*, 1992). In this report we showed that this method is reasonably accurate. The genetic interpretation of the IP-variants was confirmed by electrophoresing single individuals (Table 3.1) and the two sets of allele frequency data were within a narrow range (Table 3.2). Evidently electrophoresing single individuals is more precise but is too laborious as an adjunctive technique for breeding for resistance (Bakker *et al.*, 1992).

Modern plant nematology is confronted with the need to analyse gene products from minute amounts of biological material. As shown here the mini 2-DGE system extends the possibilities of biochemical research in plant nematology. Mini 2-DGE allows the analysis of single individuals, but obviously can also be applied to other problems in plant nematolgy where a high sensitivity is required.

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Chapter 4

Changes in two-dimensional protein patterns during post-embryonic development of *Globodera rostochiensis*

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Summary

Miniature two-dimensional gel electrophoresis was used to examine the proteins of successive post-embryonic developmental stages of the potato cyst-nematode, *Globodera rostochiensis*. Major changes in protein pattern occured during the transformation of preparasitic second-stage juveniles into parasitic juveniles and during the metamorphosis of male juveniles into adults. The relative abundance of three muscle proteins was used to demonstrate the breakdown and restoration of the body-wall musculature during these two developmental events. The protein patterns of male juveniles contained 15 abundant spots which were absent or small in female juveniles. Four of these 15 spots were also abundantly present in embryonated eggs. It is likely that one or more of these 15 proteins are involved in the metamorphosis of juvenile males into adults. Possibly some of these proteins are storage proteins that are used for the synthesis of proteins for the adult male.

Introduction

Potato cyst-nematodes (*Globodera rostochiensis* and *G. pallida*) are obligate root parasites of solanaceous plants, which form a major threat to potato cultivation world-wide (Evans & Stone, 1977). Their life cycle can only be accomplished in intimate relationship with their host plant through the formation and exploitation of syncytial feeding cells within the roots (Jones, 1981). Because of this endoparasitic life style, the development of cyst-nematodes is characterized by

events and developmental stages that are not observed in the less specialized ectoparasitic plant-feeding nematodes.

The annual life cycle of potato cyst-nematodes begins with hatching of second-stage juveniles (J2) from cysts in the soil in response to exudates released by the roots of the host plant. These infective (or preparasitic) J2 penetrate the root epidermis, usually behind the root tip, and migrate through the cortex in search for a suitable host cell for syncytium induction (Steinbach, 1972; Steinbach, 1973). As soon as such a cell is found – usually within 3 hours after root penetration – the J2 stops migrating and becomes sedentary by breaking down its body-wall musculature (Günther & Kerstan, 1969; Günther, 1972; Steinbach, 1973). The now parasitic J2 starts feeding from this initial feeding cell, which soon merges with neighbouring root cells to form a metabolically active multinucleate syncytium (Jones & Northcote, 1972). Juveniles that are destined to become females continue to feed from the syncytium during the third and fourth juvenile stages, and during early adulthood. Their development is mainly characterized by a swelling of the body due to ovarial growth and egg production, and a concomitant thickening of the cuticle wall (Chitwood & Buhrer, 1946; Shepherd *et al.*, 1972). Juveniles that develop into males, on the other hand, stop feeding after the J3 stage. Following the moult to the fourth juvenile stage they undergo a metamorphosis during which they increase in body length and restore their body-wall musculature (Günther, 1972). After the final moult, the adult males have regained their mobility and leave the root in search for females. The embryonic development of the fertilized eggs commences within the female body, and when the female finally dies her cuticle wall forms a protective cyst which encloses a few hundred eggs with diapausing infective J2 of the next generation (Hagemeyer, 1951).

Current information about the development of parasitic juvenile stages of cyst-nematodes comes largely from microscopic studies concerning their general morphology (e.g. Chitwood & Buhrer, 1946; Raski, 1950), ultrastructure (Günther & Kämpfe, 1966; Günther, 1972; Endo, 1988; Endo & Wyss, 1992; Endo, 1993 *a*; Endo, 1993 *b*; Jones *et al.*, 1993; Jones *et al.*, 1994), rate of growth (Günther & Kerstan, 1969; Müller *et al.*, 1981) and feeding behaviour (Steinbach, 1973; Wyss, 1992; Böckenhoff & Grundler, 1994). Information about the biochemical characteristics of these parasitic developmental stages is virtually lacking. In a previous paper we used conventional two-dimensional gel electrophoresis to examine the proteins of four developmental stages of *G. rostochiensis* (De Boer *et al.*, 1992 *a*; Chapter 2). Only a single sedentary juvenile stage, the J4 female, was included then. Here we extend this previous analysis to the complete range of post-embryonic developmental stages by using a much more sensitive miniature

2D-electrophoresis technique (De Boer *et al.*, 1992 *b*; Chapter 3). Emphasis is put on the developmental changes in protein pattern that occur during the transformation of preparasitic J2 into sedentary J3 and during the metamorphosis of J3 males into adults. By following the variations in abundance of three major muscle proteins, the degeneration and restoration of the body-wall musculature has been monitored during these two events.

Materials and methods

Nematodes

Globodera rostochiensis population Ro_1-mier was grown on potato plants as described (De Boer *et al.*, 1992 *a*; Chapter 2). Preparasitic second-stage juveniles (J2) were isolated by hatching cysts in potato root diffusate as described in Chapter 6. Adult females and adult males were isolated from roots of infected potato plants as described (De Boer *et al.*, 1992 *a*; Chapter 2). For the collection of parasitic second-stage juveniles (J2P) roots were harvested 14 days post-inoculation. Following processing of the root balls in a blender the root homogenate was washed through a series of sieves of decreasing pore size and the 22-180 μm fraction was collected. This suspension was further purified by centrifugation (1000 *g*; 5 min) in a solution of 35% sucrose which was covered with a layer of tap water, after which the nematodes could be collected from the sucrose-water interface. The sample thus obtained contained approximately 2% motile J2, 74% immobile, slender, slightly curved J2P (Fig. 4.1 A) and 24% thickened J2P (Fig. 4.1 B). After removal of remaining fragments of root tissue, such as small root tips, this nematode suspension was used directly for preparing the protein sample. Parasitic third-stage juveniles were isolated from infected roots about 19 days post-inoculation using the same procedure as for J2P, with the difference that now the 100-250 μm fraction was collected. Late third-stage females (J3F) and late third-stage males (J3M) were handpicked from the nematode sample under a dissecting microscope to prepare separate protein samples. The sexes were identified by their body shape (Raski, 1950): J3F become gradually tapered towards their anterior end (Fig. 4.1 C) whereas J3M remain essentially cylindrical over their entire body length (Fig. 4.1 F). Only juveniles of which the sex could be identified with certainty using these criteria were collected. The isolation of fourth-stage juveniles was performed 4 weeks post-inoculation and followed the same procedure as for J3. The protein sample of female J4 (J4F) contained a mixture of slender and swollen juveniles (Fig. 4.1 D,E). The protein sample of fourth-stage males (J4M) was made from juveniles which had completed the elongation phase (Fig. 4.1 G). Embryonated

eggs containing developed J2 were isolated from brown cysts 3 months post-inoculation. These cysts had been kept moist to prevent desiccation of the eggs. The cysts were gently pressed open in a small glass mortar filled with tap water, and the eggs were washed out of the cysts by rinsing the suspension with a Pasteur pipette. The eggs were separated from the cyst walls by centrifugation in 35% sucrose and used for sample preparation. In a similar way, eggs in approximately the 1-8 cell stage were isolated by crushing fully grown white females that were collected six weeks post-inoculation..

The time expired between the separation of the parasitic juveniles from the roots with the blender and the moment of their actual homogenization was in all cases over 4 h. This will have given the nematodes ample time to defecate (Wyss, 1992) so that it can be safely assumed that the protein samples of feeding juveniles did not contain proteins of host plant origin. For adult females it has already been demonstrated that the host plant has no influence on the two-dimensional protein pattern (Bakker & Bouwman-Smits, 1988).

Electrophoresis

After washing the nematode developmental stages in 10 mM Tris-HCl, pH 7.2, protein samples were made by homogenization in 60 μl of 10 mM Tris-HCl, pH 7.2, supplemented with 5% ß-mercaptoethanol, followed by the addition of 64 mg urea (De Boer *et al.*, 1992 *a*; Chapter 2). In cases when only a limited quantity of nematode material was available, the homogenizations were performed directly in 20-30 μl of urea sample buffer (De Boer *et al.*, 1992 *b*; Chapter 3). Each protein sample was prepared from at least 200 individuals to exclude any influences of genetic variation on the protein patterns. The protein concentrations of the samples were determined according to Bradford (1976). Mini 2D-electrophoresis followed by silver staining was performed as described (De Boer *et al.*, 1992 *b*; Chapter 3). Approximately 5 μg of protein were applied per focusing gel. This quantity corresponds to approximately 630 embryonated eggs, 1000 J2, 500 J2P, 150 J3F, 80 J3M, 82 J4M, 24 J4F, 66 males or 3 females.

Western blotting

For the identification of nematode muscle proteins, total protein extracts of infective J2 were separated by mini 2D-electrophoresis. The gels were electroblotted onto PVDF membrane and the blots were stained with primary antibody and alkaline phosphatase conjugated secondary antibody (see Chapter 6). The following primary antibodies were used: (1) a monoclonal antibody which recognizes an epitope that has been conserved in all actins ranging from human skeletal muscle to plants (Cat. no. 69-100-1; ICN Biomedicals, Costa Mesa,

USA); (2) monoclonal antibody 5.23 raised against *Caenorhabditis elegans* paramyosin, which was a kind gift from H.F. Epstein, Baylor College of Medicine, Houston, USA (Ardizzi & Epstein, 1987); and (3) monoclonal antibody MGR 3, which binds to the body-wall muscle filaments of infective J2 of *G. rostochiensis* (Chapter 6).

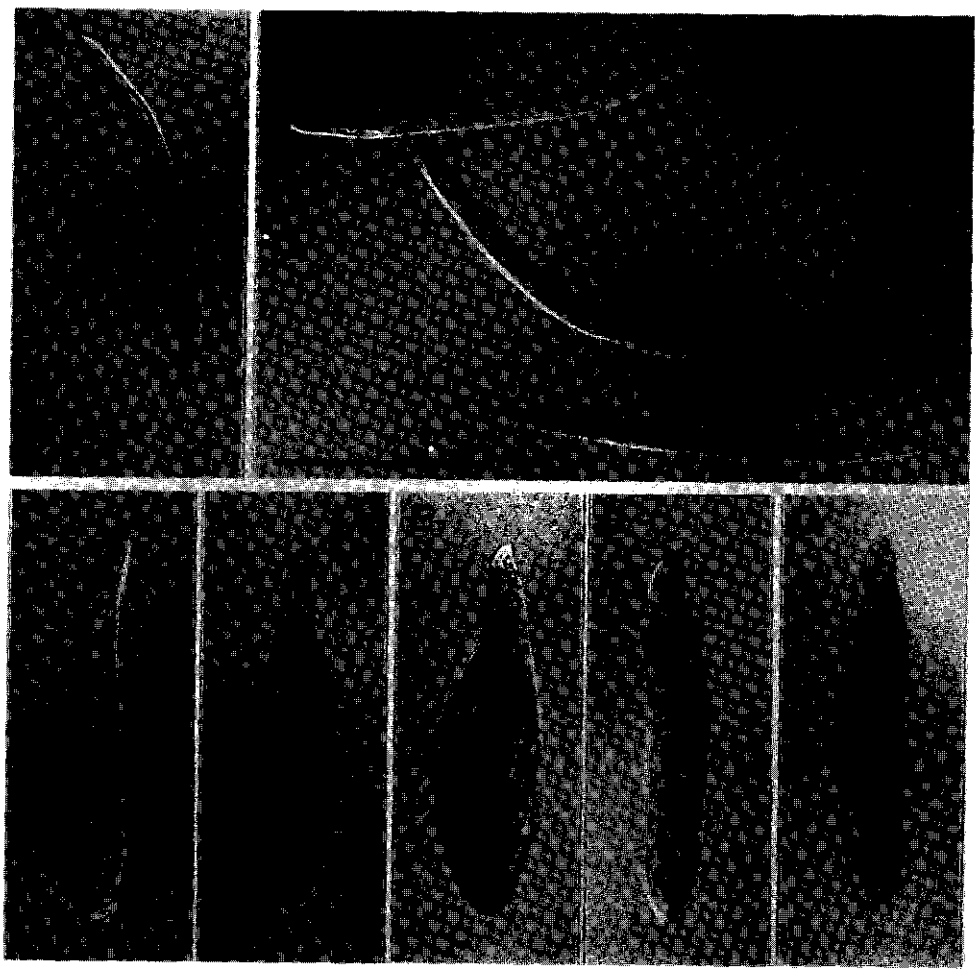

Fig. 4.1. Parasitic developmental stages of *Globodera rostochienis* used for preparing the protein samples. **A, B.** parasitic J2; **C.** J3 female; **D, E.** J4 females; **F.** J3 male; **G.** J4 male. All micrographs were taken from formaldehyde-fixed, glycerin-mounted nematodes, using interference contrast optics. Scale bar = 200 μm.

Spot analysis

The 2D-gels were dried between sheets of cellophane using a vacuum gel dryer, and compared on a view box using a loupe. To assess the number of changing protein spots between two developmental stages counts were made of disappearing and newly appearing spots, and also of spots that showed a marked increase or decrease in size between the two stages. The percentage of changing spots was calculated using the formula $2n_{xy}/(n_x+n_y)$, with n_x and n_y being the number of spots observed in stages X and Y respectively, and n_{xy} the number of changing spots between both stages. The analysis was confined to a single well-stained gel for each developmental stage, with the occasional consultance of one or two other gels when necessary. Where possible protein spots were given the same identification numbers as in a previous analysis of conventional 2D-gels (De Boer *et al.*, 1992*a*; Chapter 2), otherwise they were assigned a new number between 1000 and 2000. The abundance of protein spots was estimated by measuring their area on the original gels with a scale loupe (De Boer *et al.*, 1992*a*; Chapter 2). Conspicuous proteins which were also resolved in conventional 2D-gels of *G. rostochiensis* were used as internal standards to estimate the pH gradient and molecular weights.

The handling of the protein spot data was greatly facilitated by using a self-written computer program (MINISPOT), which could read digital images of the electrophoresis patterns and store information about the protein spots in these images as it was collected during the analysis of the gels. This computer program, which runs under the TOS operating system (Atari Corporation, Sunnyvale, USA), is available upon request. The protein patterns shown in this chapter are all digitized images which were obtained by scanning black and white photographs of the original gels.

Results

Identification of body-wall muscle proteins

To monitor the changes in body-wall muscle mass during juvenile development, major muscle proteins were identified in the two-dimensional protein pattern of preparasitic J2 by immunoblotting. Staining with a monoclonal antibody to actin resulted in a very strong reaction with protein spots 362, 363, and 364 (Fig. 4.2 A). Additional staining occured of small protein spots of increasing molecular weight above spot 363 (small arrows) and a small spot previously assigned number 702 (De Boer *et al.*, 1992 *a*; Chapter 2). The horizontal streak in the electrophoresis pattern is the result incomplete electrofocussing of actin that has remained on the top (T) of the first-dimension gel. Anti-paramyosin showed a

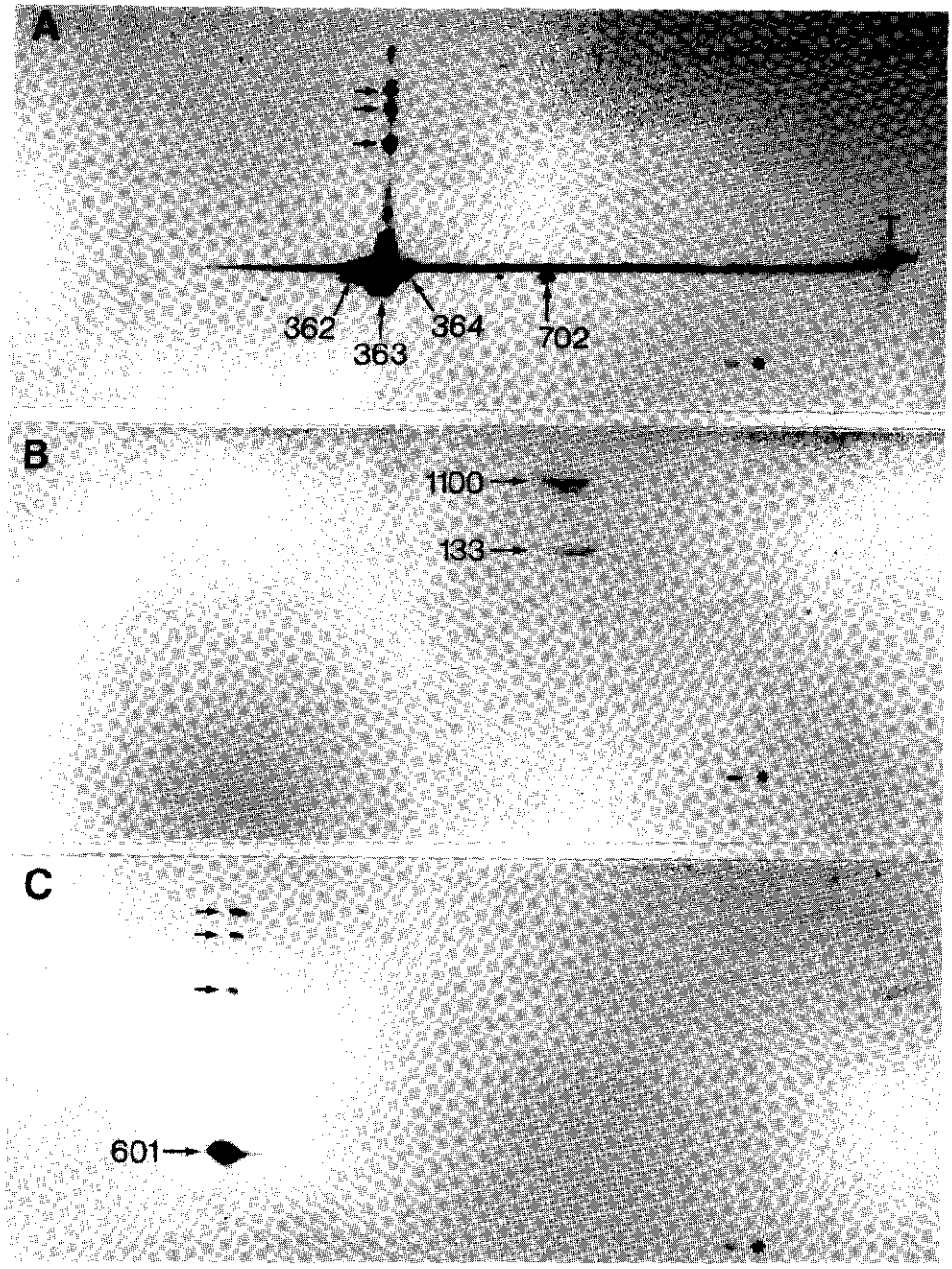

Fig 4.2. Immunodetection of muscle proteins on two-dimensional Western blots of preparasitic J2 of *G. rostochiensis*. **A.** Reaction with a monoclonal antibody against actin. **B.** Reaction with a monoclonal antibody against paramyosin. **C.** Reaction with monoclonal antibody MGR 3 against the body-wall muscle filaments of *G. rostochiensis*. The asterisks indicate a small spot which is stained non-specifically by the akaline phosphatase conjugate in each of the Western blots. For further explanation see main text.

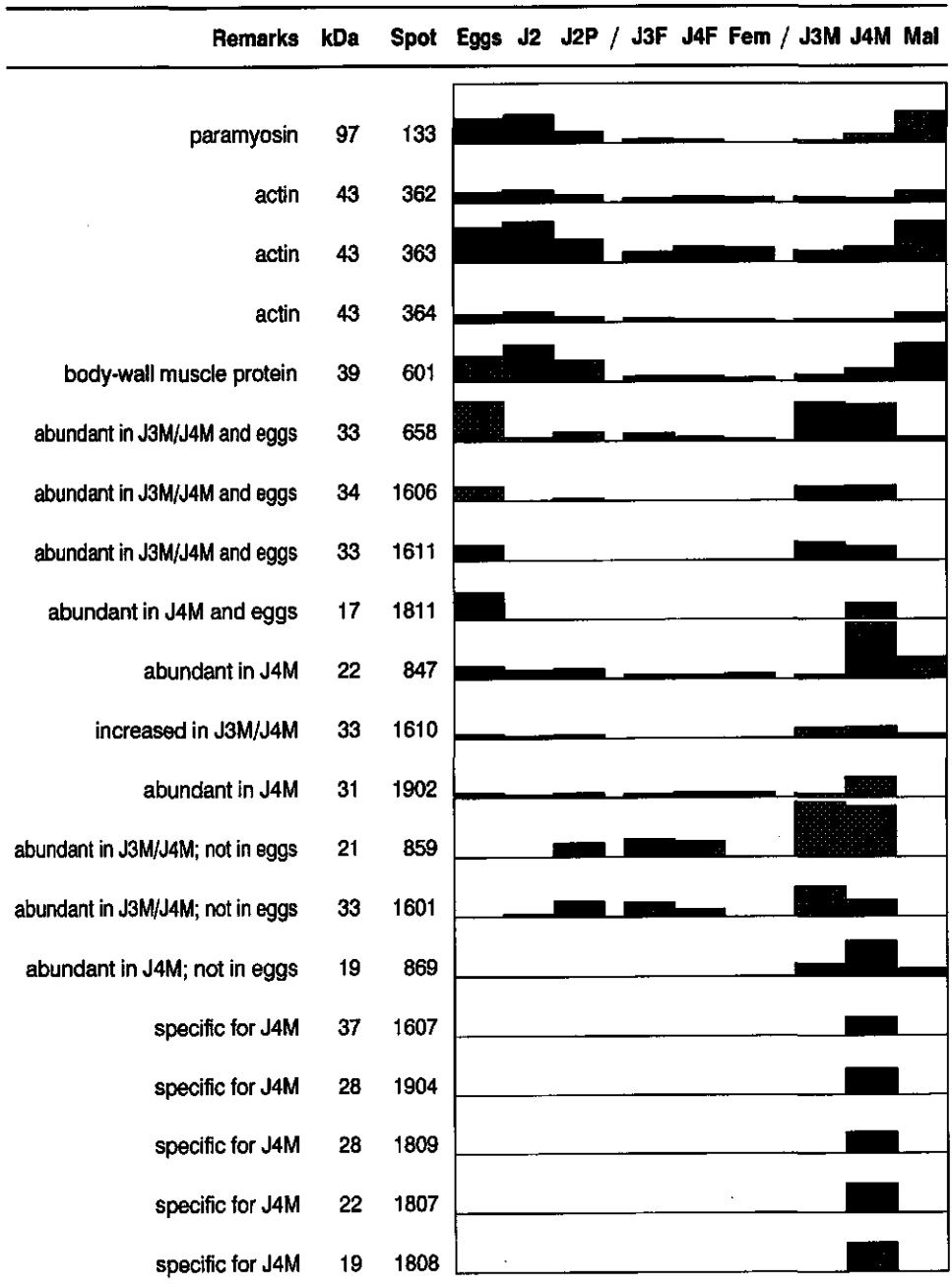

Fig. 4.3. Changes in the dimensions of selected protein spots in two-dimensional gel electrophoresis patterns of *G. rostochiensis*. The size of each spot in each developmental stage is indicated with a bar, on a scale from 0 to 6.5 mm^2 (see Table 4.1 for abbreviations).

moderate reaction with spot 133 and a strong reaction with a very high molecular weight spot (labelled 1100) of the same isoelectric point (Fig. 4.2 B). Monoclonal antibody MGR 3, specific for body-wall muscle filaments in J2 of *G. rostochiensis*, showed intense staining of spot 601 (Fig. 4.2 C). Also here, minor spots with higher molecular weights were stained as well (small arrows). The changes in spot dimensions of actin (362, 363, 364), paramyosin (133, 1100) and muscle protein 601 observed in the 2D-gels of the successive developmental stages are presented in Fig. 4.3.

Transformation into sedentary parasites

The transformation of preparasitic J2 into sedentary juveniles is illustrated by the protein patterns of preparasitic J2 (Fig. 4.4), parasitic J2 (Fig. 4.5) and J3 females (Fig. 4.6). In preparasitic J2 107 proteins were found which disappeared or showed a large decrease in size in the protein patterns of parasitic juveniles. For some of these proteins this decline already happens in the parasitic J2 stage, while for others it is delayed until the J3 stage. Among the decreasing spots are paramyosin (133/1100), the actin isomers (362-364) and muscle protein 601 (Fig. 4.4). Only the central actin isomer (363) remains present in J3 as a fairly abundant spot. Complementary to this phenomenon is the appearance of 103 protein spots in J3 females that are either absent or much smaller in size in preparasitic J2 (Fig. 4.6). A large proportion (58%) of these newly appearing spots was also observed in embryonated eggs (see below), and therefore their value as marker proteins for the sedentary stages remains uncertain. The number of changing spots between J2 and J2P (141) is comparable with the number of spots that change from J2P to J3F (132) or to J3M (135) (Table 4.1). A direct comparison of J2 with J3F showed that no less that 214 protein spots (40.5%) change in size or presence during the transformation to the sedentary phase (Table 4.1).

Development of females

The development into females is illustrated by the protein patterns of J3F, J4F and females (Figs. 4.6-4.8). In these gels no further evident changes are seen in the abundance of either paramyosin, spot 601 or actin (Fig. 4.3). While the moult of J3F into J4F involves relatively few changes (10.4%) in protein composition, the differentiation of J4F into adult females again shows a large proportion (34.2%) of changing spots (Table 4.1). To trace proteins that can be considered characteristic for the feeding juvenile stages, the gels of J3F, J3M, and J4F were compared against the non-feeding J4 males. This identified a single medium sized spot (# 1901), which is small in preparasitic J2, males and females, and an additional smaller spot which is present only in J3 and J4F (Fig. 4.6).

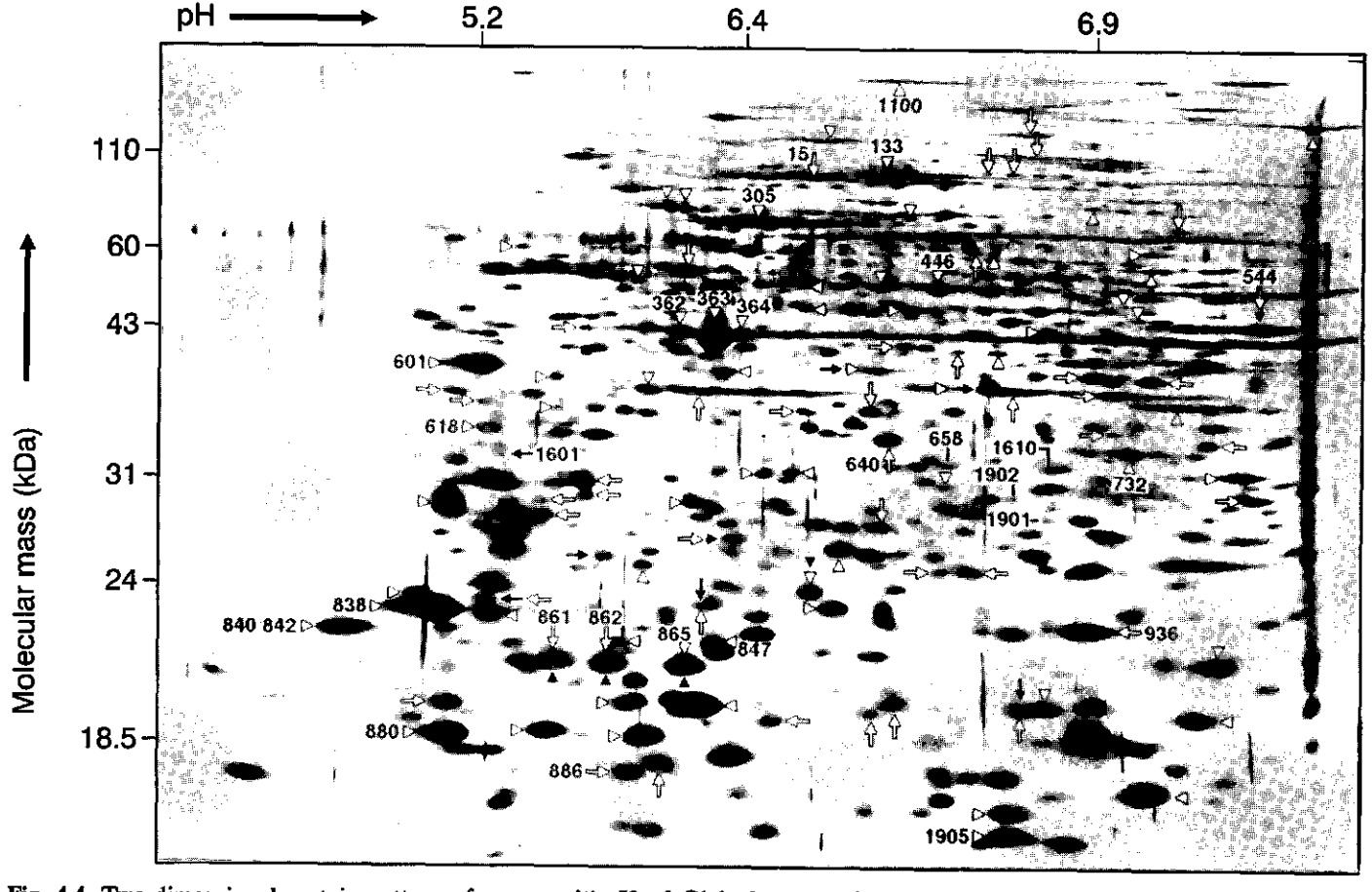

Fig. 4.4. Two-dimensional protein pattern of **preparasitic J2** of *Globodera rostochiensis*. *White arrows* indicate spots that disappear or decrease in size in parasitic J2. *White arrowheads* indicate spots whose disappearance or maximum decrease is postponed until the J3 stage. Also marked are spots that are new (*black arrows*) or larger (*black arrowheads*) compared with embryonated eggs.

Table 4.1 - Numerical comparison of two-dimensional protein patterns of *G. rostochiensis*. Presented are the percentage (above diagnonal) and the number (below diagonal) of changing spots between pairs of developmental stages. N_{tot}: total number of spots/gel. (Eggs: embryonated eggs. J2: preparasitic second-stage juveniles. J2P: parasitic second-stage juveniles. J3F, J4F: third- and fourth-stage female juveniles. J3M, J4M: third- and fourth-stage male juveniles. Fem: adult females. Mal: adult males).

	N_{tot}	Eggs	J2	J2P	J3F	J4F	Fem	J3M	J4M	Mal
Egg	511		16.5							
J2	481	82		27.0	40.5			42.8		
J2P	564		141		23.1			24.7		
J3F	575		214	132		10.4		5.4		
J4F	544				58		34.2			
Fem	508					180				
J3M	529		216	135	30				13.7	
J4M	511							71		36.5
Mal	475								180	

Development of males

The development into males is illustrated by the protein patterns of J3 males (Fig. 4.9), J4 males (Figs. 4.10 and 4.11) and males (Fig. 4.12). A comparison of the electrophoresis patterns of J3 females and J3 males showed very few differences (5.4%) between these two stages (Table 4.1). The most notable differences were found in J3 males, which possess a few large spots that are either not present in J3 females (1606, 1610, 1611) or much increased in size compared with J3 females (658, 859).

Although the percentage of spots that change from J3 males to J4 males is much less (13.7%) compared with the changes observed in the preceeding developmental stages (Table 4.1), the electrophoresis pattern of J4 males is characterized by a series of proteins (Figs. 4.3, 4.10). Some of these J4M-dominant spots are already abundantly present in J3 males (e.g. 658, 1606, 1611, 859, 1601), while others are new or much increased in size in J4 males (e.g. 1811, 847, 1902, 869, 1607, 1904, 1809, 1807, 1808). Fig. 4.3 gives an overview of the occurrence of these J4M-dominant spots in the other developmental stages. Twenty spots of J4 males could not be traced any of the other stage examined, and are thus unique to this developmental stage (Fig. 4.10).

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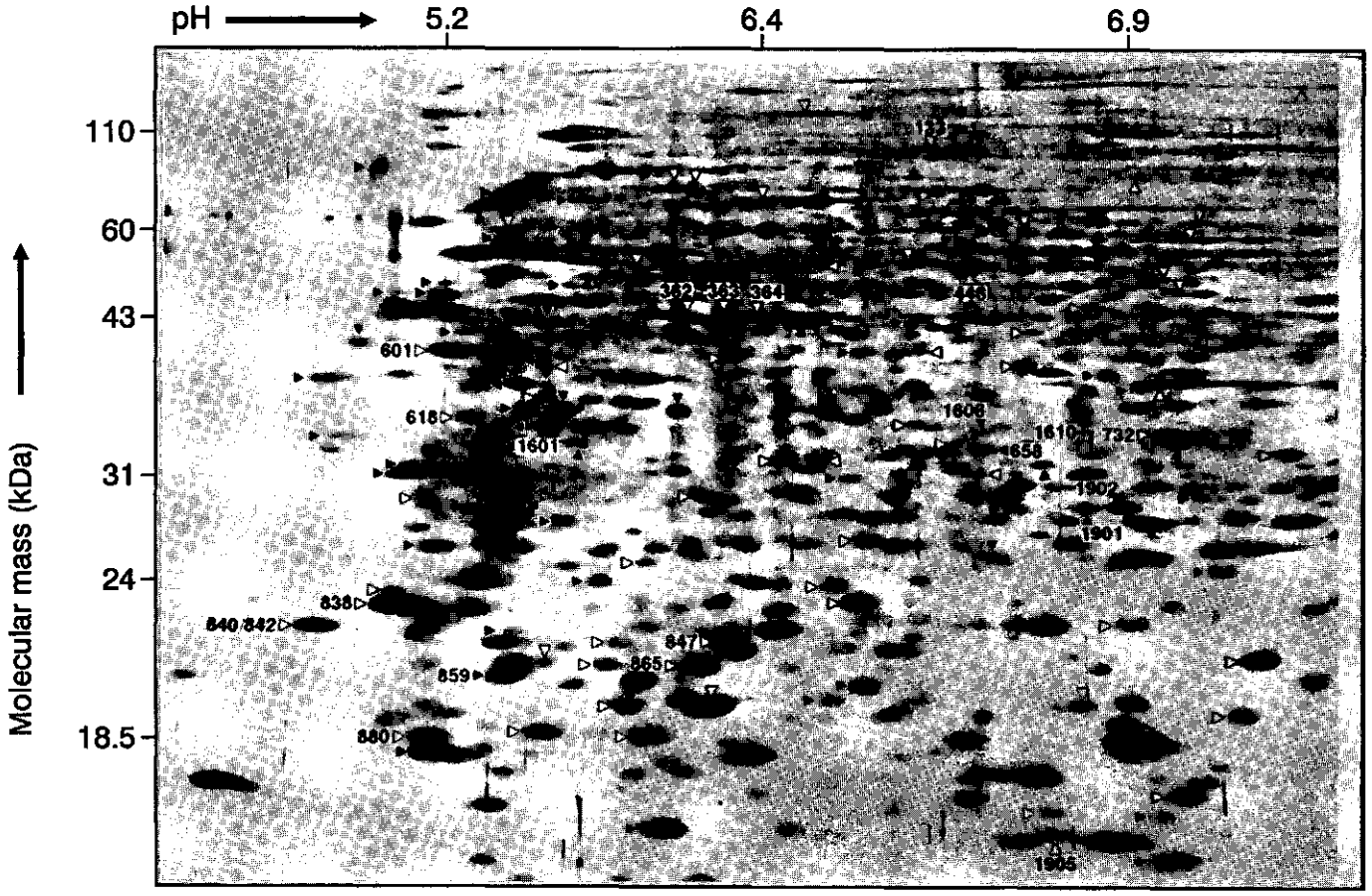

Fig. 4.5. Two-dimensional protein pattern of **parasitic J2** of *Globodera rostochiensis*. The transformation of the J2 into sedentary juveniles is illustrated by the presence of spots from preparasitic J2 that disappear or decrease in the J3 stage (*white arrowheads*) and by the first appearance of spots that are new or increased in parasitic juveniles (*black arrowheads*). Spots that reach a maximum size in parasitic J2 are boxed.

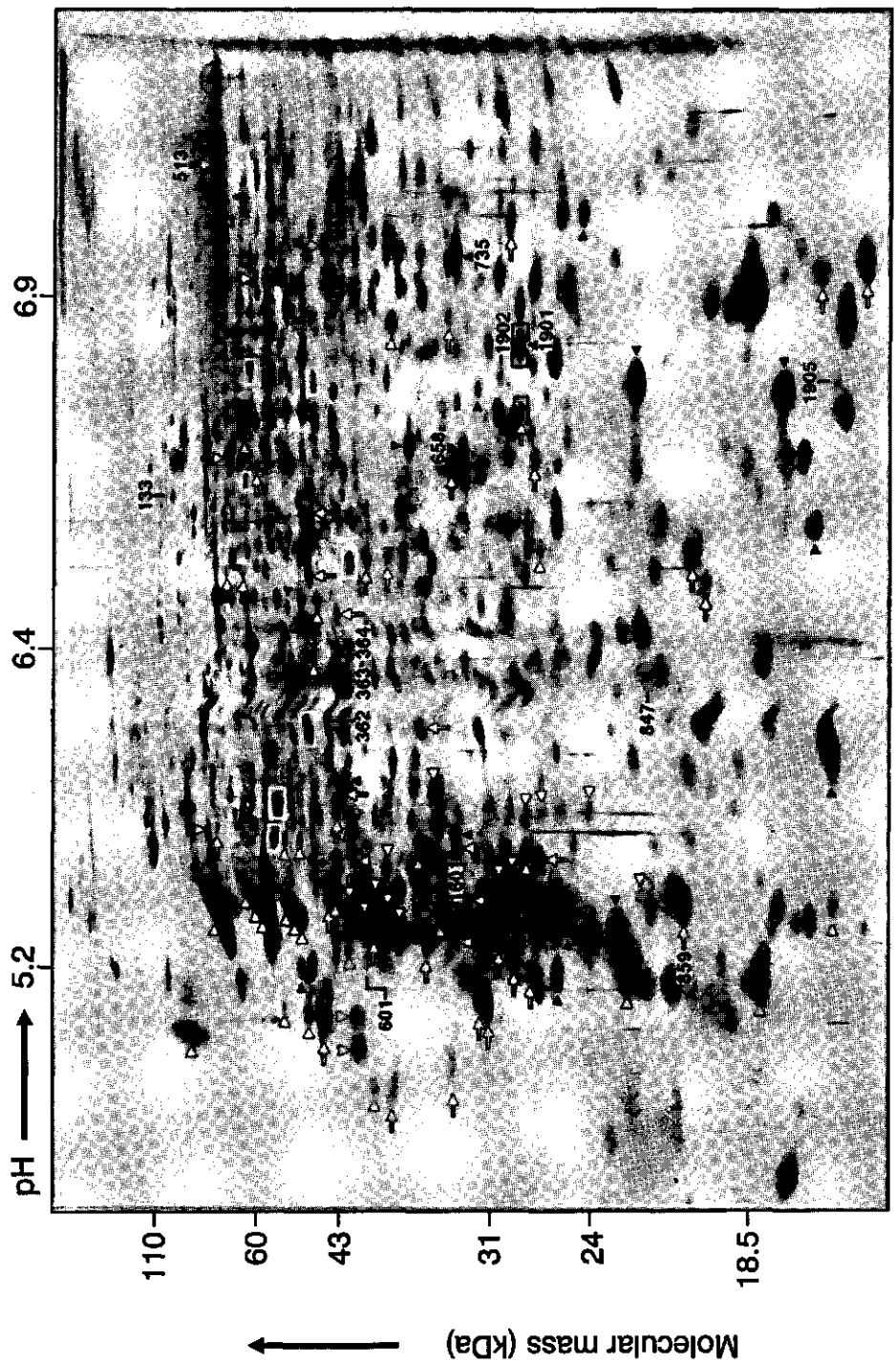

Fig. 4.6. Two-dimensional protein pattern of **J3 females** of *Globodera rostochiensis*. *White arrows* indicate spots which are absent in preparasitic J2 and also absent in embryonated eggs. *White arrowheads* indicate spots which are absent in preparasitic J2 but present in embryonated eggs. *Black arrowheads* indicate spots which are increased in size compared with preparasitic J2. *Black boxes* indicate spots that are characteristic for feeding juveniles. *White boxes* indicate spots that disappear in J4 females.

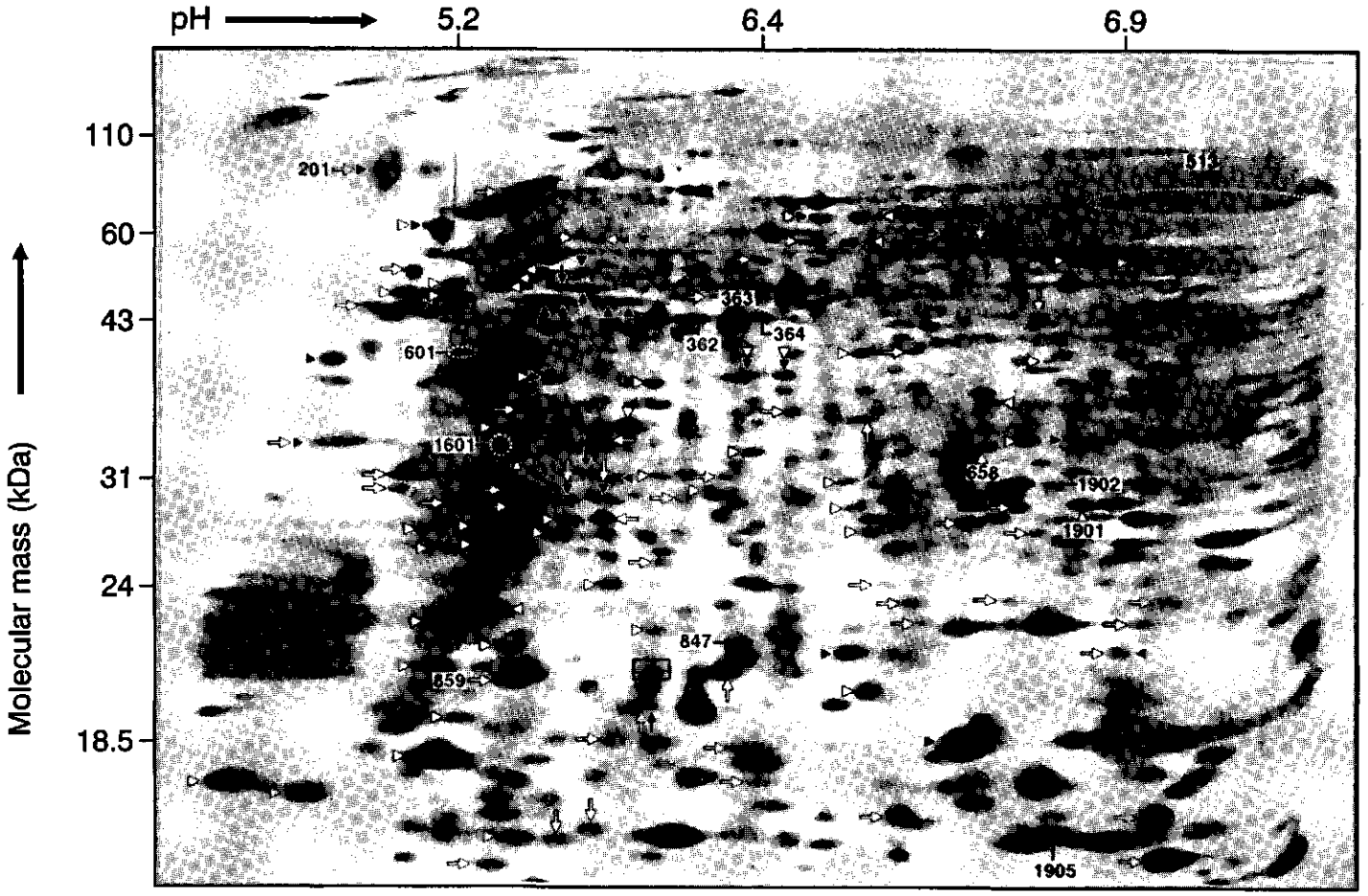

Fig. 4.7. Two-dimensional protein pattern of **J4 females** of *Globodera rostochiensis*. *Black arrows*: not in J3 females. *Black arrowheads*: larger than in J3 females. *White arrows*: disappearing in adult females. *White arrowheads*: decreasing in adult females. *Black boxes* indicate spots that are present only in J4 females.

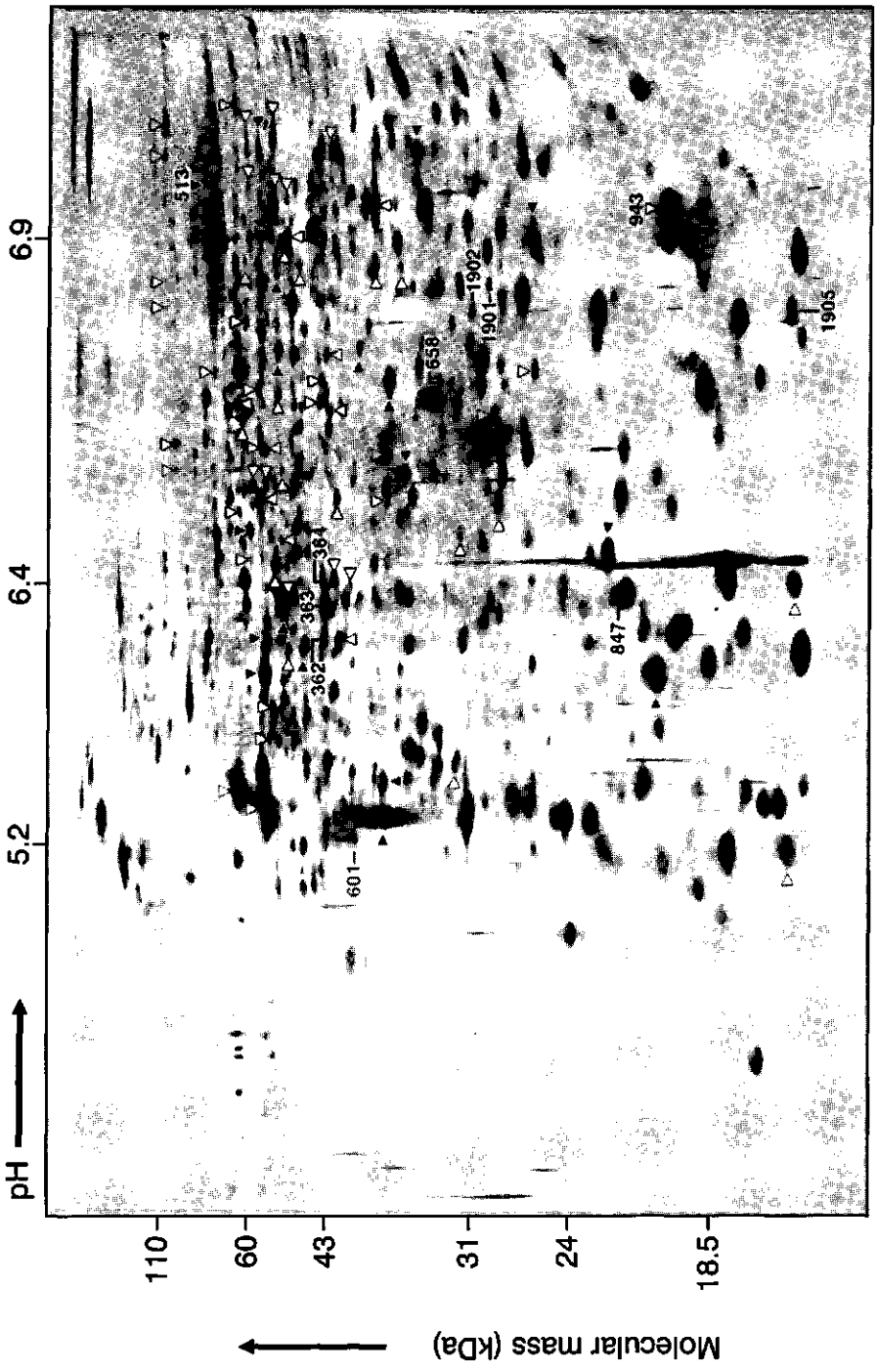

Fig. 4.8. Two-dimensional protein pattern of **adult females** of *Globodera rostochiensis*. *White arrowheads*: not in J4 females. *Black arrowheads*: larger than in J4 females.

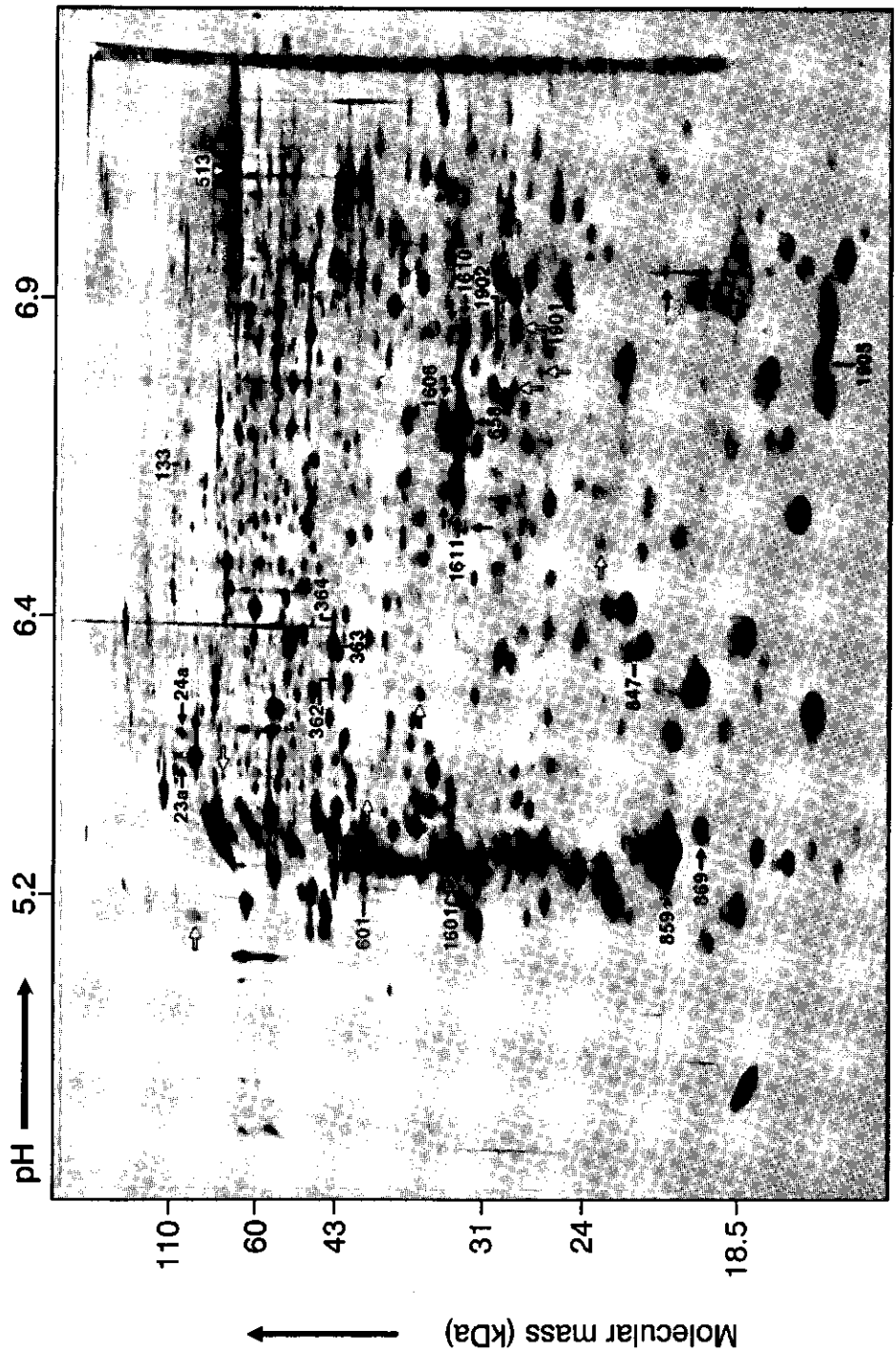

Fig. 4.9. Two-dimensional protein pattern of **J3 males** of *Globodera rostochiensis*. *Black arrows*: not in J3 females. *Black arrowheads*: larger than in J3 females. *White arrows*: disappearing in J4 males. *White arrowheads*: decreasing in J4 males.

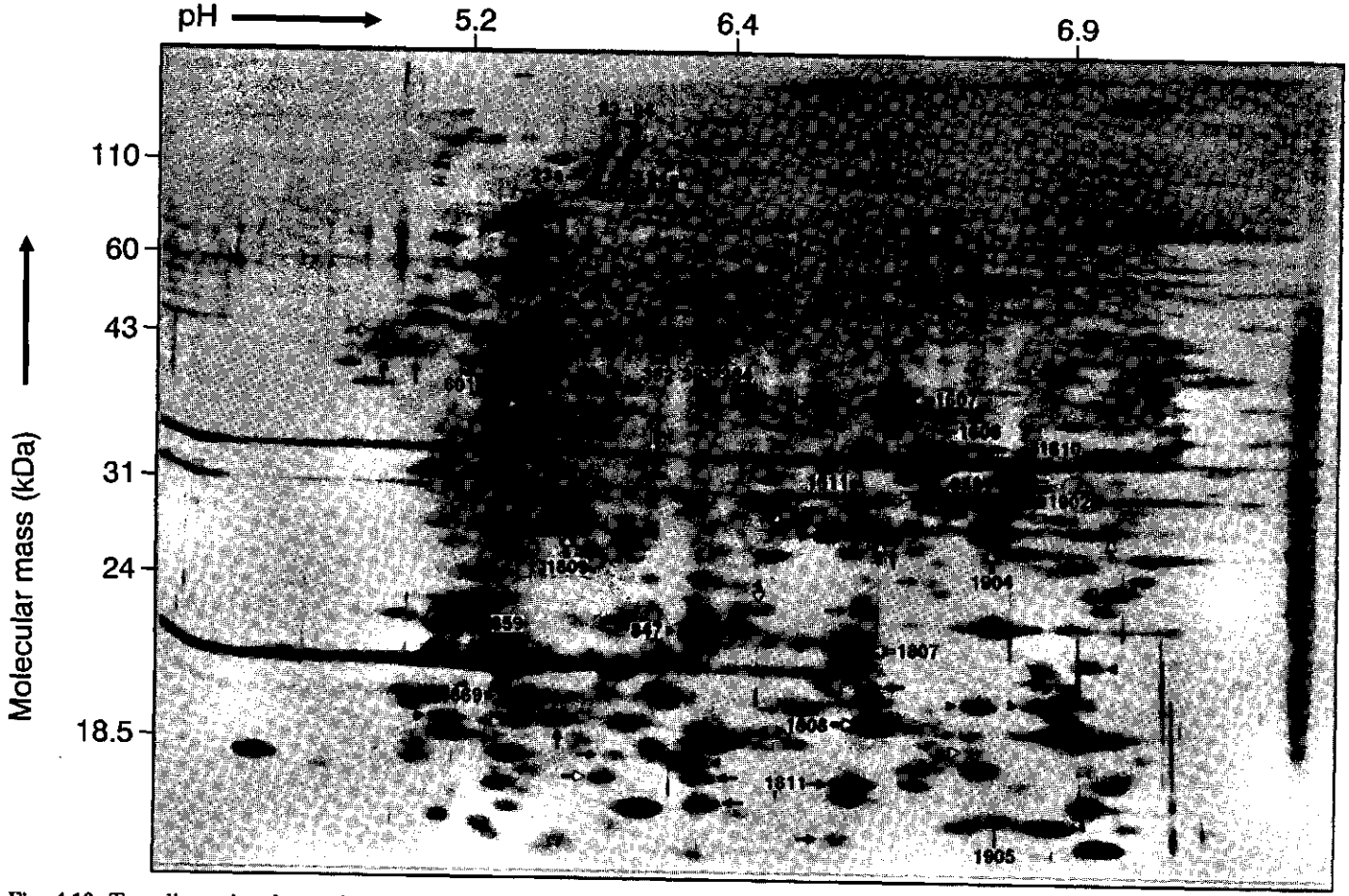

Fig. 4.10. Two-dimensional protein pattern of **J4 males** of *Globodera rostochiensis*: differences with J3 males. *White arrows*: spots specific for J4 males (only 18 of 20 are indicated). *Black arrows*: absent in J3 males, but present in one or more other stages. *Black arrowheads*: larger than in J3 males.

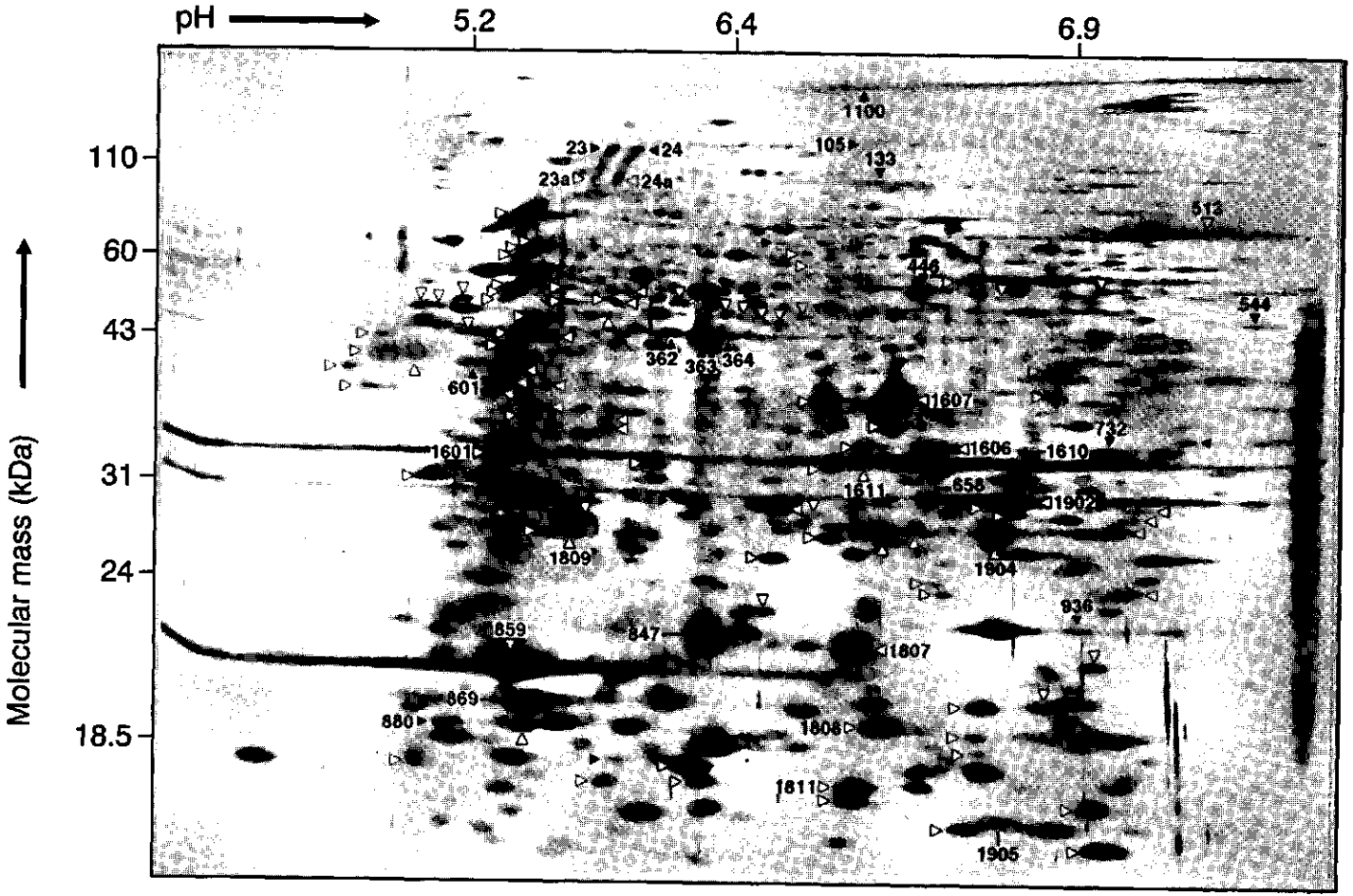

Fig. 4.11. Two-dimensional protein pattern of **J4 males** of *Globodera rostochiensis*: differences with adult males. *White arrowheads*: disappearing in males. *Black arrowheads*: increasing in males.

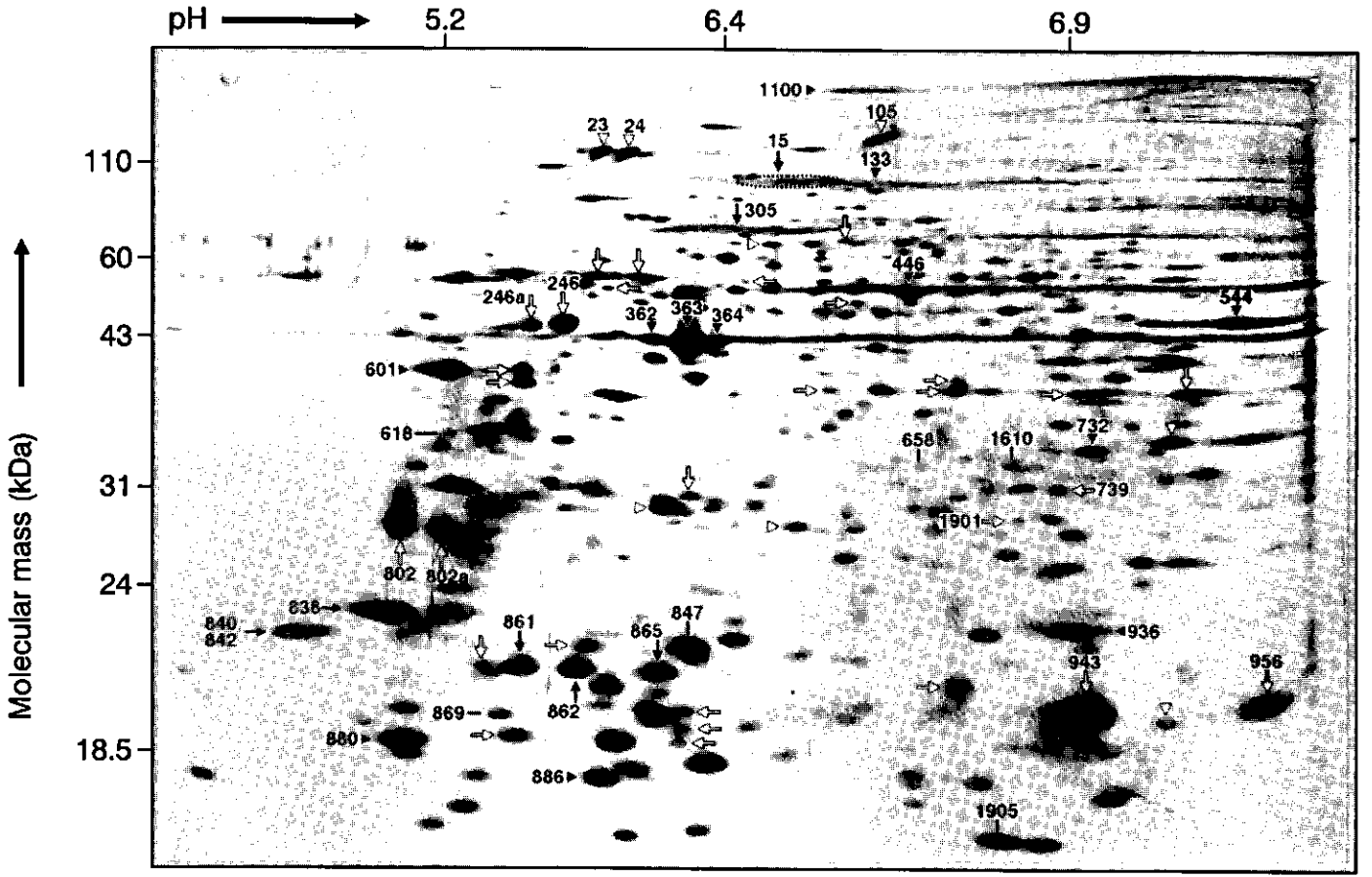

Fig. 4.12. Two-dimensional protein pattern of **adult males** of *Globodera rostochiensis*. *Arrows*: absent in J4M. *Arrowheads*: larger than in J4M. Arrow(head)s in *black* indicate dominant spots from preparasitic J2 which disappear or decrease during the transformation to parasitic juveniles, and which return or regain their original abundance in males.

(text continued from page 71)

The transformation of J4 males into adult males again shows a considerable change in the protein pattern (Figs. 4.11 and 4.12), which is not only documented by the spot counts (Table 4.1), but also illustrated by a dramatic change in the dominant protein spots. The large spots that are characteristic for parasitic males all disappear or show a sharp decrease in size in adult males (Fig. 4.3) and they are replaced by a new set of dominant spots (Fig 4.12). Several of these new large spots were also abundantly present in preparasitic J2 and they now reach a similar abundance again in males. Among these 'returning' spots are actin, paramyosin, and muscle protein 601 (Fig. 4.3). Although these muscle proteins already show a slight increase in spot size in J4M, their full return does not occur until the males have become adults. Males also show proteins that are unique (spots 246, 246a, 802, 802a, 739, and 956) or most abundant (spots 23, 24, 105 and 943) in this stage. Spots 23 and 24 are already clearly visible in J4 males, where they interconnect with a corresponding set of spots (23a and 24a) of lower molecular weight (Fig. 4.10). These latter spots are already visible in the J3 males (Fig. 4.9). Spot 943 was not found in any of the other developmental stages, except for females (Fig. 4.8) where it is much decreased in size.

Eggs

Because it was suspected that the dominant proteins accumulating in male juveniles might be storage proteins, we also looked for these proteins in eggs. In young eggs of the 1-32 cell stage none of the J4M-dominant spots were found (not shown). However, in eggs containing fully developed J2, four large spots (658, 1606, 1611 and 1811) were present in quantities similar to those observed in J4 males (Figs. 4.3 and 4.13). In addition 5 smaller spots occurring in parasitic males were also retrieved in eggs. Remarkable was the observation that embryonated eggs possess several spots which disappear in preparasitic J2, and reappear in parasitic juveniles of both sexes (Fig. 4.13). Characteristic abundant proteins of embryonated eggs are spots 640 and 1905. Spot 640 decreases in J2 and J2P, and is not present in any of the other stages. Spot 1905 is present in almost the same size in young eggs, and in decreased dimensions in all other stages. Actin, paramyosin and muscle protein 601 are already present in embryonated eggs in quantities close to those observed in preparasitic J2 (Fig. 4.3).

Fig. 4.13. Two-dimensional protein pattern of **embryonated eggs** from cysts of *Globodera rostochiensis*. *White arrows*: disappearing in preparasitic J2 and returning in male juveniles. *White arrowheads*: disappearing in preparasitic J2 and returning in parasitic juveniles of both sexes. *Black arrows*: disappearing in preparasitic J2 and remaining absent in subsequent stages. *Black arrowheads*: decreasing in preparasitic J2 and remaining small (or disappearing) in subsequent stages. The dotted line indicates the perimeter of spot 601, which was stained very weakly in this gel.

Discussion

The microscopic size of parasitic juveniles of cyst-nematodes makes it difficult to collect these stages in sufficient quantities for protein analysis by conventional 2D-electrophoresis. In this chapter we applied a 10-fold more sensitive miniature 2D-electrophoresis technique (De Boer *et al.*, 1992 *b*; Chapter 3) and were thus able to produce high quality protein patterns from all parasitic juvenile stages of *G. rostochiensis*. This allowed the examination of two major events in the development of cyst-nematodes, namely the transition of infective J2 into sedentary juveniles and the metamorphosis of juvenile males into adults.

Identification of muscle proteins

To be able to monitor the changes in body-wall musculature during cyst-nematode development, we first sought firm identification of three major muscle proteins in the protein patterns of *G. rostochiensis*. In a previous paper (De Boer *et al.*, 1992 *a*; Chapter 2) we tentatively identified actin (spots 362, 363, 364), paramyosin (spot 133) and tropomyosin (spot 601) on the basis of their molecular mass and electrofocussing position. Here the identification of actin and paramyosin has been confirmed by immunostaining of Western blots of J2. The identification of spot 601, however, remains incomplete. Although it did react with a monoclonal antibody to the body-wall musculature of *G. rostochiensis*, attempts to stain this spot with two commercially available antibodies to chicken gizzard tropomyosin failed. An unexpected reaction of anti-paramyosin antibody 5.23 occurred with a protein spot of more than 200 kDa (spot 1100). We also observed this staining reaction in one-dimensional SDS-electrophoresis patterns of J2 of *G. rostochiensis* (data not shown). A similar high molecular weight band is visible in electrophoresis patterns of paramyosin from *Caenorhabditis elegans* (Harris & Epstein, 1977; Kagawa *et al.*, 1989), and it can therefore be assumed that spot 1100 represents an undissociated aggregate of paramyosin molecules. Likewise, the additional small spots of increasing molecular weight that were seen on the Western blots stained for actin and spot 601 may represent undissociated multimeres of these proteins.

Changes in muscle proteins

The changes in abundance of actin, paramyosin and spot 601 have provided detailed information about the presence of muscle fibres in the various developmental stages of *G. rostochiensis*. Since body-wall muscle cells constitute the largest muscle tissue component of nematodes (Anderson, 1989), the changes in abundance of these muscle proteins in the electrophoresis patterns are closely

related to the changes in body-wall muscle tissue mass in the developmental stages. The protein patterns of parasitic J2 and J3 show that the degeneration of the body-wall muscle cells already begins in the second-stage parasitic juveniles and is probably completed in the late J3-stage. This quick decline of the muscle proteins is in agreement *in vivo* microscopic observations, which have shown that J2 of cyst-nematodes become immobile as soon as they have initiated their feeding cell (Steinbach 1973; Wyss, 1992). During the development of female juveniles into adults the muscle proteins show no further changes in their abundance. The fairly abundant central actin isform (363) that remains present in these stages probably originates from cytoplasmic actin filaments rather than from muscle tissue (Bretscher, 1991). However, it has been reported that a limited amount of body-wall muscle cells remains present in the neck region of females (Shepherd & Clark, 1978) and also at other locations such as the anus, vulva, and pharyngeal region muscle cells can be expected to remain present (Anderson, 1989). During the metamorphosis of J3 males to adults the body-wall musculature is fully restored. In J4 males only a slight increase was noticed in the muscle proteins. This means that the assembly of muscle fibres must occur essentially in the very late J4M stage, well after the elongation of the juvenile within the shed J3 cuticle. This conclusion is confirmed by microscopic observations of the beet cyst-nematode (*Heterodera schachtii*) in which the actual restoration of male body-wall musculature has been found to occur during the moult of the J4 male to adult (Günther, 1972).

Abundant spots in parasitic males

In parastic males a remarkable accumulation of several abundant proteins was observed. Based on their distribution in the other developmental stages these spots can be divided into two categories: (1) those that are abundant exclusively in J4 males and (2) those that are also abundantly present in one or more of the other developmental stages. It is likely that a number of these abundant proteins fulfill an essential role in the metamorphosis of J4 males to adults. In holo-metabolous insects the pupal metamorphosis is mediated by storage proteins which have accumulated in the fat body during the feeding period of the preceeding larval stages (Levenbook, 1985). It is possible that cyst-nematodes use an analogous system of storage proteins to manage their complex life cycle. The most likely candidates for such a function are proteins from the second category which show an accumulation in the feeding J3 males (e.g. spots 658, 1601, 1606, 1611 and 869). The appearance of new abundant proteins in J4 males, however, is not easily explained because at this stage the juveniles do not feed any more. It has been reported for *Heterodera schachtii* that during the elongation phase of J4

males the inner surface of the shed J3 cuticle is gradually dissolved (Günther & Kämpfe, 1966; Günther 1972). It was proposed that this process served a dual function, namely to weaken the J3 cuticle so that the adult males can escape from it, and to provide a source of materials to be used anew in the metamorphosis process (Günther & Kämpfe, 1966). It is therefore possible that the abundant spots occurring specifically in the protein sample of J4 males are breakdown products from the J3 cuticle. In that case spot 513 could well be a cuticle protein which is broken down during the metamorphosis, since it is abundantly present in J3 males (Fig. 4.6) and considerably less abundant in J4 males (Fig. 4.10).

The presence of spots 658, 1606 and 1611 in embryonated eggs may indicate that processes requiring utilization of storage proteins also take place during hatching of preparasitic J2. In hatched J2 these proteins have disappeared. Instead, actin, paramyosin, and muscle protein 601 have slightly increased in abundance, and also a few other a few spots have increased in size or are new in J2 compared with embryonated eggs.

A reverse process may be expected at the onset of parasitism, where the surplus of amino acids released from the breakdown of body-wall muscle filaments may require storage in the form of proteins. Parasitic J2 indeed show the appearance of spots 859 and 1601, and an increase in spot 658. While in female juveniles the presence of these spots is only temporary, in parasitic males they are retained and show a further increase in abundance.

Differentiation into males

The differentiation of J4 males into adults is accompanied by a radical change of the two-dimensional protein pattern. In adult males the abundant proteins of J4 males are no longer present and they have been exchanged for a new set of large protein spots, which includes the major muscle proteins. The appearance of these new spots can only be explained by assuming that several (if not all) of the disappearing J4M proteins have been used for *de novo* protein synthesis in males. The protein pattern of males showed several spots (23, 24, 105, 246, 246a, 739, 802, 802a, 943, 956) that are characteristic or unique for this stage and it is possible that some of these proteins originate from the testis. Interesting in this respect is that spots 23 and 24 can already be seen in J3 males, where they appear to be present as the faster migrating spots 23a and 24a. Since the development of the testis commences in the third juvenile stage (Chitwood and Buhrer, 1946; Raski, 1950), this may indicate that spots 23 and 24 are associated with the testis throughout the development of the male stages.

General conclusions

The numerical comparison of the electrophoresis patterns (Table 4.1) has shown that the most extensive changes in protein composition occur during the transition of preparasitic J2 into sedentary juveniles (up to 42.8%), during the metamorphosis of parasitic males (36.5%), and also during the differentiation of J4 females into adults (34.2%). While the latter developmental process predominantly involves changes in minor protein spots, the former two events are accompanied by major shifts in dominant protein spots, and can therefore be considered the most dramatic steps in the development of *G. rostochiensis*. Similar extensive developmental changes in protein expression have been found in the animal parasitic nematode *Haemonchus contortus*, where a difference of 68% was observed between the two-dimensional protein patterns of free-living J3 and parasitic J4 (Kooyman & Eysker, 1995).

The developmental pathway from preparasitic J2 to adult males in *G. rostochiensis* is an extraordinary example of the flexibility with which parasitic nematodes can recycle their proteins in order to complete the different phases of their life-cycle. Following root penetration, the migratory infective J2 transform into a sedentary parasitic stage, and after feeding for only about one week (Steinbach 1973) they undergo essentially a reversal of this initial transformation process in which they re-adapt to a migatory mode of life. Proteins that are specific to the motile stages (De Boer *et al.*, 1992 *a*; Chapter 2) are temporarily broken down in the feeding J2 and J3, but subsequently synthesized again in adult males, utilizing the protein resources that have been acquired during the short feeding period as a parasitic juvenile. Several of these major 'returning' spots have been marked in Fig. 4.12), and their appearance in the protein pattern of males is evidence that processes functionally similar to the metamorphosis of insects take place during the metamorphosis of cyst-nematodes.

In all, the protein analysis presented in this chapter has confirmed our previous conclusion based on selected developmental stages (De Boer *et al.*, 1992 *a*; Chapter 2) that potato cyst-nematodes display an extremely dynamic protein metabolism during their life-cycle. Although at the moment we can only speculate upon the function and identity of most of the protein spots visible in the electrophoresis patterns of *G. rostochiensis*, it will be a more than worth while subject for future research to further elucidate the developmental changes in protein expression described here.

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Chapter 5

Protein analysis of microdissected second-stage juveniles and males of *Globodera rostochiensis*

Jan M. de Boer & Hein A. Overmars

Summary

Anterior and posterior body parts of preparasitic second-stage juveniles (J2) and adult males of the potato cyst-nematode *Globodera rostochiensis* were collected by microdissection of individual nematodes in a hypertonic solution. The protein composition of these nematode fragments was examined with mini 2D-electrophoresis. In the anterior parts of both J2 and males an acidic protein spot of 34.4 kDa was identified, which was virtually absent in the corresponding posterior parts. It is concluded that this protein originates from cells or structures that are specific to the anterior body part of these developmental stages. A number of protein spots that are characteristic of males of *G. rostochiensis* were abundanlty present in the posterior sections of males, and absent or only weakly visible in the anterior sections. It is concluded that several of these male-characteristic spots may originate from the testis.

Introduction

In preparasitic J2 of *G. rostochiensis*, oesophageal gland secretory proteins are synthesized in two subventral gland cells and one dorsal gland cell, where they are stored in secretory granules. In the subventral glands these secretory granules take up almost the entire volume of the gland cell (Chapter 7, Fig. 7.5), whereas in the dorsal gland they occupy only about 25% of the cell volume and have accumulated at the anterior pole of the cell (Chapter 1, Fig. 1.4). In adult males of *G. rostochiensis* the three oesophageal glands are probably also filled with secretory granules. It has been shown that the extension of the dorsal gland contains electron dense granules of the same type that is present in J2 (Walsh *et al.*, 1983). Also interference contrast microscopy of live males of *G. rostochiensis* shows that the extensions of both the dorsal and the subventral glands are swollen due to an accumulation of secretory granules (J.M. de Boer, personal

observations). More detailed studies with other cyst-nematodes have demonstrated that all three oesophageal glands are active in males, and that they contain the same types of secretory granules as observed in preparasitic J2 (Baldwin *et al.*, 1977; Endo, 1984; Wyss & Zunke, 1986).

In this chapter, live preparastic J2 and males of *G. rostochiensis* have been cut in anterior parts (containing the oesophageal glands) and posterior parts (without the oesophageal glands), and the proteins of these nematode fragments have been analysed by mini 2D-electrophoresis with the aim to identify putative oesophageal gland secretory proteins.

Materials and methods

Microdissection

Preparasitic J2 and adult males of *G. rostochiensis* were collected as described in Chapter 2. To prepare the nematodes for microdissection, they were first incubated at room temperature for 1-1.5 h in a hypertonic buffer (derived from Powers *et al.*, 1986) containing 168 mM sucrose, 480 mM mannose, 50 mM HEPES-NaOH pH 7.4 and 10 mM EDTA. The density of this buffer is just below the density of the nematodes (so that they do not float to the fluid surface) while the osmolarity is sufficiently high for a gradual withdrawal of water from the animals. This ultimately causes a slight shrinkage of the nematodes and neutralizes the hydrostatic pressure of their body fluids: the nematodes can now be dissected easily without the risk of extrusion of body contents.

After this 'equilibration period' in hypertonic buffer the nematodes were cut in anterior and posterior parts under a dissecting microscope, using a fragment of a razor blade attached to a plastic pipette tip. The location of the transverse cut is shown in Fig. 5.1: the J2 were cut in halves, whereas the males were cut in the region between the oesophageal glands and the anterior end of the testis. The nematodes remained in hypertonic buffer (at room temperature) during sectioning. The nematode sections were immediately transferred to separate dishes with hypertonic buffer of 0 °C, using a glass Pasteur pipette of which the tip had been narrowed to about 0.25 mm. Approximately 200 front and rear fragments were collected over a period of 3 h. The fragments were centrifuged to the bottom of a 1.5 ml microcentrifuge tube (5 min at 10,000 *g*), and excess buffer was withdrawn until a volume of 30 μl remained on top of the nematode sections. Then 32 mg urea and 6 to 7 μl β-mercaptoethanol were added, and the sample was stored at -80 °C until further processing. Aliquots of nematode fragments from both males and J2 were thus collected and frozen during several days until enough material was available for electrophoresis.

Electrophoresis

To prepare protein samples for electrophoretic analysis, eight anterior and posterior samples derived from 1400 dissected males and sixteen anterior and posterior samples derived from 3300 dissected J2 were thawed. A mixture (6 μl) containing 25% 2-mercaptoethanol, 8% ampholytes pH 5-7 and 2% ampholytes pH 3-10 was added to each thawed sample to dissolve all urea. The samples were then pooled and the nematode fragments were spun down in a 1.5 ml microcentrifuge tube. Most of the supernatant buffer (500-1000 μl) was removed and concentrated to approximately 25 μl in a 1.5 ml microcentrifuge filter concentration unit with a molecular mass cut-off of 10 kDa (Ultrafree-MC, Millipore Corp., Bedford, USA) by centrifugation at room temperature at 8000 *g* for 1 to 2 h. The nematode fragments, which were left in 25 μl buffer, were homogenized in a small glass mortar. After this, the concentrated supernatant was added to the nematode homogenate, and this sample was used for electrophoretic analysis. Mini 2D-electrophoresis followed by silver staining was performed as described in **Chapter 3.**

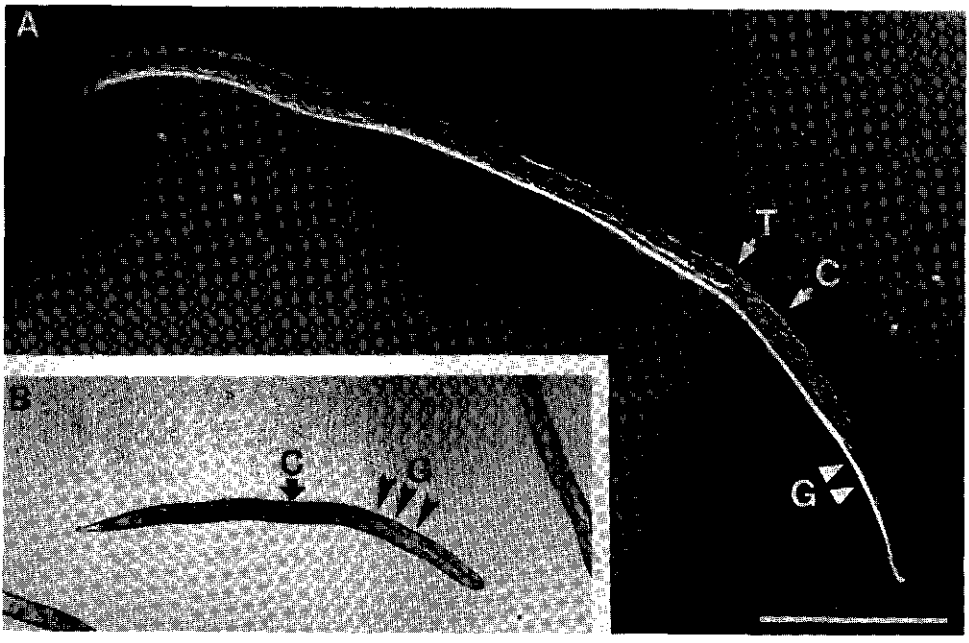

Fig. 5.1. Developmental stages used for microdissection. **A.** Adult male. **B.** Second-stage juvenile. C, region of transverse cut; G, region of the oesophageal glands; T, anterior end of the testis. Bar = 200 μm.

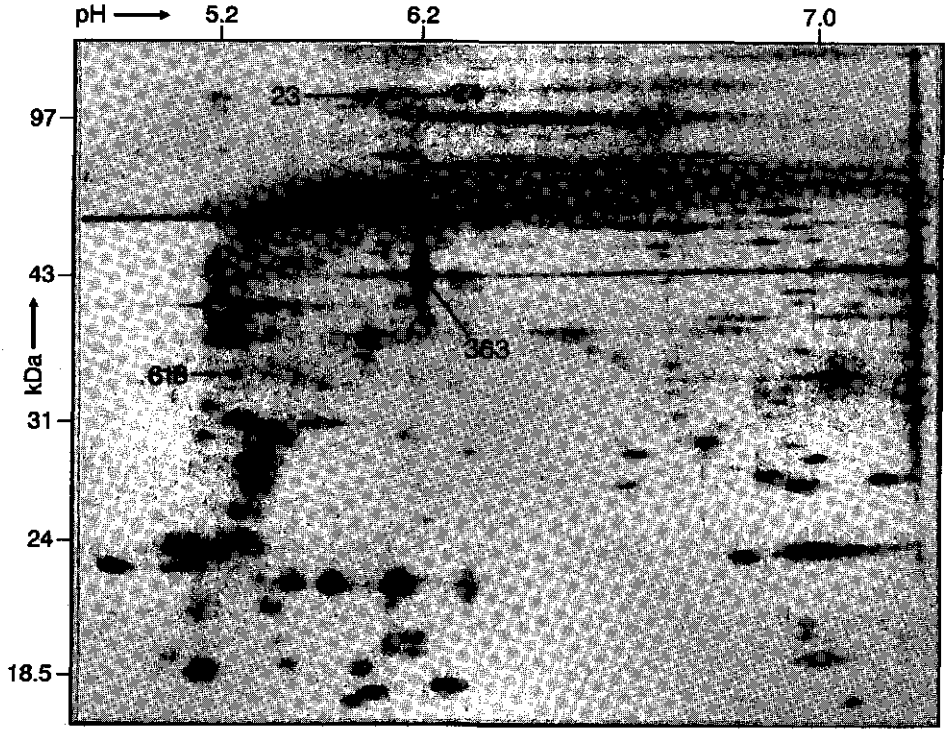

Fig. 5.2. Two-dimensional electrophoresis pattern of 448 anterior parts of males of *Globodera rostochiensis* (the quantity of protein applied to this gel was about 3.0 μg). Spot 618 is not visible in the corresponding posterior parts (Fig. 5.3).

Results and Discussion

The protein samples from anterior and posterior portions of males had produced acceptable 2D-patterns in which respectively 149 and 186 protein spots could be distinguished. This is respectively 31% and 39% of the number of spots that can be seen in a typical 2D-pattern of a total protein homogenate of males (cf. Chapter 4; Table 4.1). The anterior portions of males showed only one spot (no. 618) which was not seen in the posterior portions (Fig. 5.2). Conversely, the posterior portions showed several spots which were absent or smaller in the anterior portions (Fig. 5.3). The spots 23, 24, 105, 246, 246a, 802, 802a and 943 are abundant proteins characteristic of male developmental stages of *G. rostochiensis* (Chapter 4) and they are also abundantly present in the posterior portions of males. Apart from spots 23 and 24, these male-characteristic spots are

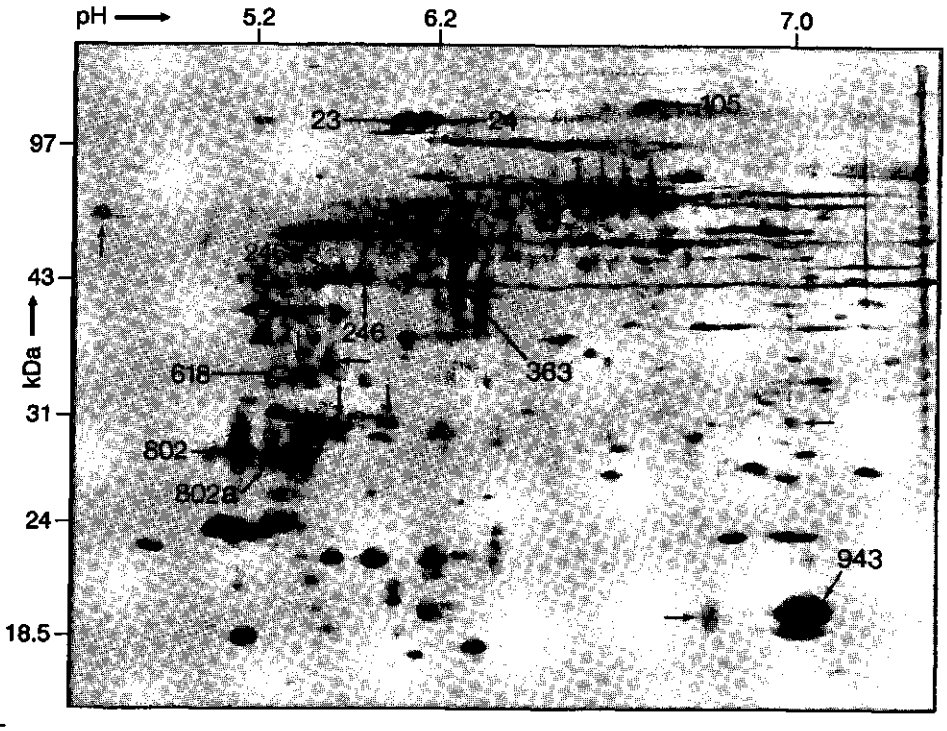

Fig. 5.3. Two-dimensional electrophoresis pattern of 140 posterior parts of males of *Globodera rostochiensis* (the quantity of protein applied to this gel was about 6.1 μg). Several spots which are not seen in the anterior parts of males (Fig. 5.2) are indicated with arrows. Spot 618 is not visible in this gel.

completely absent in the anterior parts of the males. This indicates that spots 105, 246, 246a, 802, 802a and 943 may orgininate from the testis, which is located in the posterior parts of the dissected males (Fig. 5.1). The presence of minute quantities of spots 23 and 24 in the anterior sections suggests that these proteins are characteristic of the intestine. However, it cannot be excluded that the anterior sections have become slightly contaminated with material from the testis during microdissection, and that spots 23 and 24 are of testis origin. Spot 943 is not strictly male-specific, since it can also be retrieved (in much decreased concentration) in homogenates of adult females of *G. rostochiensis* (Chapter 4). This may indicate that this protein is specific to the gonads of both sexes.

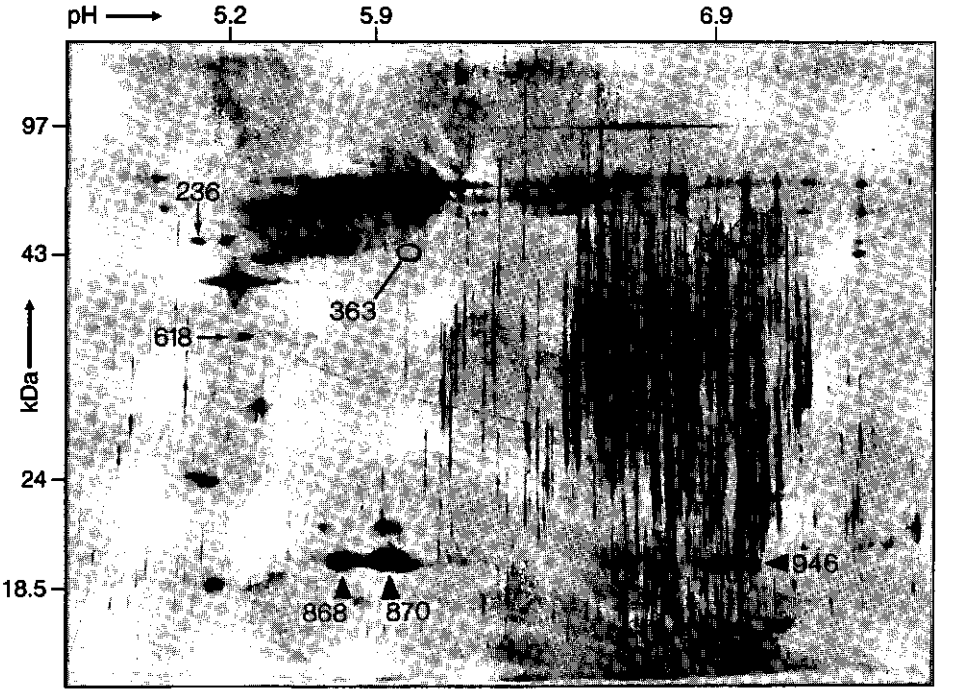

Fig. 5.4. Two-dimensional electrophoresis pattern of 3300 anterior parts of second-stage juveniles of *Globodera rostochiensis*. In the posterior parts (Fig. 5.5) spot 236 is not present and spot 618 is barely visible. The arrowheads indicate spots which are increased in size compared with the posterior parts. The vertical stripes are artifacts of electrophoresis.

Following electrophoresis of the dissected J2 very few protein spots were visualized with the silver stain (Figs. 5.4 and 5.5) indicating that a considerable loss of proteins had occurred during sample preparation (cf. Chapter 4, Fig. 4.4). The absence of actin isoform 363 in the anterior portions of J2 illustrates the severe incompleteness of this electrophoresis pattern, and this means that little or no value can be attached to the few spots that appear to be specific to the posterior parts of the J2 (Fig. 5.5). Nevertheless, the anterior sections of J2 showed two spots, labelled 236 and 618, which were either not seen (236) or only faintly visible (618) in the posterior sections (Fig. 5.4).

The 2D-patterns of both J2 and males have shown that spot 618 is characteristic of the anterior body parts of these developmental stages. The molecular

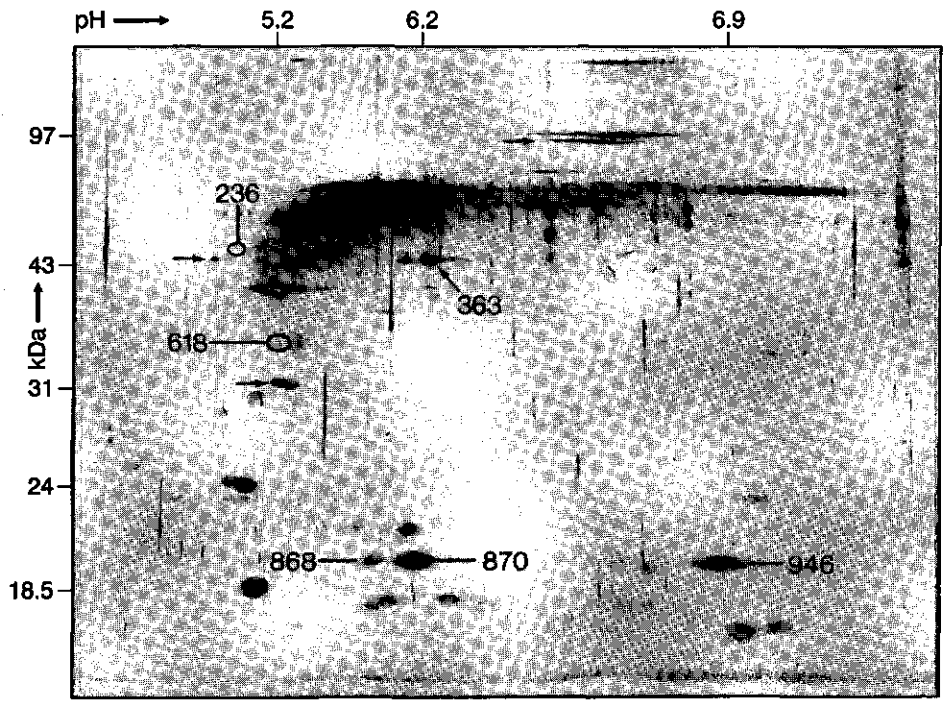

Fig. 5.5. Two-dimensional electrophoresis pattern of 3300 posterior parts of second-stage juveniles of *G. rostochiensis.* Arrows indicate spots not seen in the anterior portions.

mass of this protein is estimated to be 34.4 kDa and its pI is about 5.22 (Chapter 2). It can be concluded that protein 618 is almost exclusively present in a cell type or other structure that is specific to the anterior body region of J2 and males. One plausible explanation is that spot 618 is a component of the dorsal or subventral oesophageal glands. However, alternative explanations for the origin of spot 618 are equally plausible, such as a muscle protein isoform which is expressed only in certain types of muscle cells (e.g. Ardizzi & Epstein, 1987; Kagawa *et al.*, 1995), or a protein characteristic of the amphids (Stewart, 1993). The very weak presence of spot 618 in the posterior parts of J2 may indicate that either the expression of this protein is not entirely fore-part specific, or that the posterior parts have become contaminated with material from the anterior parts during microdissection.

In the two-dimensional protein patterns of the successive post-embryonic developmental stages of *G. rostochiensis* (Chapter 4) spot 618 has its maximum

size in embryonated eggs and preparasitic J2, becomes very small in the J3 stage, remains small in adult males, and dissappears in 4th-stage females and adult females. This behaviour is exactly what can be expected of a protein that is specific to the anterior region of a cyst-nematode: due to the progressive growth of the tissues in the posterior region of the later developmental stages, the proteins from the anterior region are gradually diluted in the total protein homogenates, and thus become smaller or disappear in the electrophoresis patterns of the later stages.

To determine whether spot 618 is a protein from an oesophageal gland, this spot was isolated from 2D-gels of homogenized J2 and used for monoclonal antibody production following methods described in Chapter 6. This, however, did not produce antibodies which reacted with the oesophageal glands, and therefore the precise origin of spot 618 remains unclear.

Although also spot 236 was specific to the anterior portions of J2, this could not be confirmed with the anterior portions of males because spot 236 is not present in adult males (Chapter 2). Because of the poor quality of the electrophoresis patterns of the dissected J2, it therefore remains uncertain whether spot 236 is really fore-part specific.

The microdissection procedure used a 2.4-fold concentrated version of a buffer which was developed for the isolation of mitochondria (Powers *et al.*, 1986). It was assumed that this buffer would leave the secretory granules within the oesophageal gland cells intact during the dissection procedure. Yet, only a single spot was found to be characteristic of the anterior regions of both developmental stages examined. This somewhat disappointing result may have had several causes: (1) proteins may have been broken down by proteolytic enzymes following microdissection; (2) collapse of cells or cell organelles due to osmotic shock may as yet have occurred during microdissection and this may have resulted in a loss of soluble proteins from the nematode sections; (3) not all proteins of the nematode tissues may have been dissolved with the detergent-free extraction buffer used; (4) isoelectric focussing was performed in a gradient from pH 4.5 to pH 7 and therefore relevant proteins with isoelectric points outside this pH range may have escaped the analysis.

In conclusion, it has been shown that it is feasible to perform microdissection on nematodes to analyse the distribution of proteins within different body parts. The severe loss of proteins with the dissected J2 indicates that there is a lower limit to the size of the nematodes that can be analysed in this way. Based on numerous protein measurements performed for the 2D-gel analyses in Chapters 2 and 4, the protein content of individual J2 is estimated to be 5.0-5.4 ng, whereas adult males contain about 75-92 ng of total protein per individual. In view of the

acceptable protein patterns obtained from males, it can be concluded that a minimum protein content of about 75 ng/nematode is necessary for succesful microdissection analysis.

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Chapter 6

Production and characterization of monoclonal antibodies to antigens from second-stage juveniles of the potato cyst-nematode, *Globodera rostochiensis*

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Summary

Following immunization of mice with different antigens from *Globodera rostochiensis*, monoclonal antibodies (MAbs) were screened by fluorescence microscopy for reaction with specific structures in second-stage juveniles (J2) of *G. rostochiensis*. MAbs were obtained which bound to the subventral oesophageal glands, the body-wall muscle filaments, the genital primordium, the intestinal lumen, cell nuclei, and the cuticle surface. The MAbs to the subventral glands also bound to the subventral glands in J2 of *G. pallida* and *G. tabacum*, but not in J2 of *Heterodera glycines*, *H. schachtii*, *Meloidogyne hapla* or *M. incognita*. Three subventral gland MAbs reacted with a water soluble epitope in native protein extracts from J2 of *G. rostochiensis*. The MAbs to the body-wall muscle filaments also bound to the body-wall musculature in J2 of *G. pallida*, *H. schachtii*, *M. hapla*, and *M. incognita*. On Western blots of J2 of *G. rostochiensis* these MAbs reacted with two proteins of 39 kDa and >106 kDa respectively.

Introduction

Secretory products from the oesophageal glands are considered to play an important role in the formation and exploitation of feeding cells that endoparasitic nematodes induce in the roots of their host plant (Hussey, 1989 *a*). Identification of these secretory products will provide insight into the host-parasite interaction, and may also open new possibilities for endoparasitic nematode control through genetic modification of the host plant (Schots *et al.*, 1992 *a*).

Monoclonal antibodies (MAbs) have been raised against epitopes in the dorsal and subventral oesophageal glands of both the soya bean cyst-nematode *Heterodera glycines* (Atkinson *et al.*, 1988; Goverse *et al.*, 1994) and the root-knot nematode *Meloidogyne incognita* (Hussey, 1989 *b*; Hussey *et al.*, 1990; Davis *et al.*, 1992). Various immunogens have been used in these studies, such as hatched J2, unhatched J2, adult females, microdissected anterior parts of females, a sub-cellular granule fraction from J2, and stylet secretions of females. These MAbs have provided information about the developmental expression of oesophageal gland antigens in *H. glycines* and *M. incognita* (Atkinson & Harris, 1989; Davis *et al.*, 1994; Goverse *et al.*, 1994) and they have been used for the identification of secretory granule proteins in *M. incognita* (Hussey *et al.*, 1990; Ray *et al.*, 1994).

In this paper we have used immunofluorescence microscopy to identify MAbs that bind to specific structures in the potato cyst-nematode, *Globodera rostochiensis.* In the selection of immunogens, emphasis was put on putative antigens from the dorsal and subventral oesophageal glands. Five MAbs were identified which reacted with epitopes within the subventral oesophageal glands. In addition 13 MAbs were obtained which bound to various other structures in J2. The MAbs were characterized by immunofluorescence microscopy for cross-reactivity with J2 of other plant-parasitic nematode species, and with ELISA and blotting techniques for reactivity with protein extracts from J2 of *G. rostochiensis.*

Materials and methods

Nematodes

Preparasitic second-stage juveniles (J2) of *Globodera rostochiensis* pathotype Ro_1, and of *G. pallida* pathotype Pa_2 were hatched by soaking cysts in potato root diffusate on a 100 μm sieve (Clarke & Perry, 1977). Preparasitic J2 of *Meloidogyne hapla* and *Heterodera schachtii* were gifts from respectively Mrs. E. Jansen, DLO Research Institute for Plant Protection, and H. Lubberts, DLO Centre for Plant Breeding and Reproduction Research, both in Wageningen, The Netherlands. The J2 suspensions were mixed with an equal volume of 70% (w/v) sucrose in a centrifuge tube, covered with a layer of tap water, and centrifuged briefly at 1000 *g*. Purified juveniles were then collected from the sucrose-water interface with a Pasteur pipette, washed with tap water, and used for experiments. Preparasitic J2 of *G. tabacum*, *H. glycines* and *M. incognita* were obtained as described by Goverse *et al.* (1994). Parasitic juveniles of *G. rostochiensis* were isolated from roots of infected potato plants as described in Chapters 2 and 4.

Antigen preparation

Monoclonal antibodies were produced by immunizing BALB/C mice with antigen samples from *G. rostochiensis* juveniles using four different protocols:

(1) Preparasitic J2 were homogenized in phosphate buffered saline pH 7.4 (PBS) with a small glass mortar and pestle at 4 °C. The homogenate was stored at -80 °C until used. The thawed sample was injected intraperitoneally (20000 J2, 100 μg protein) with two intraperitoneal booster injections (27000 J2, 135 μg protein) after 4 weeks and 17 weeks.

(2) The first and second immunization were as described for protocol (1); the final booster injection, however, was a crude pellet fraction derived from 200 000 preparasitic J2. These J2 were taken up in homogenization buffer containing 0.20 M mannitol, 0.07 M sucrose, 0.05 M HEPES-NaOH pH 7.5, and 0.01 M EDTA (Powers *et al.*, 1986). Portions of the suspension were spread on a large microscope slide and the nematodes were chopped into small pieces with a razor blade attached to a vibrating (50 Hz) aquarium air pump. The homogenate was filtered through a 10 μm sieve at 4 °C, and the filtrate was centrifuged for 5 min at 8000 *g*. The pellet was frozen in homogenization buffer. For immunization, the thawed pellet was suspended in 50 μl PBS and injected intraperitoneally.

(3) Female fourth-stage juveniles (J4) were fixed for 3 days in 0.2% paraformaldehyde in PBS at 4 °C. Then their anterior portions were cut off with a razor blade at about 1/4 of the juvenile's body length. Forty-nine anterior sections were thus collected in PBS. The sections were pelleted by centrifugation in a microcentrifuge tube. The supernatant was removed, and the sample was frozen at -20 °C until used. The thawed sample was homogenized in 30 μl PBS and used for intrasplenic immunization.

(4) A sodium dodecyl sulphate (SDS) extracted protein homogenate of 200 000 preparasitic J2 was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a 9% acrylamide separating gel (size 160 × 135 × 1.5 mm) and 4% acrylamide stacking gel (De Boer *et al.*, 1992 *a*; Chapter 2). After electrophoresis, a narrow zone of high molecular weight proteins was excised for electroelution, starting at the border of the separating gel and the stacking gel, and ending 1.5 mm below in the separating gel. The apparent molecular masses of the proteins in this gel segment were >200 kDa. The proteins were eluted from the gel pieces in a Model 422 Electro Eluter (Bio-Rad, Hercules, USA). The gel pieces were placed in an elution buffer containing 25 mM Tris, 192 mM glycine, 0.1% SDS, and a current of 50 mA was applied for 18 h at 4 °C. The eluted proteins were trapped in membrane caps with a molecular mass cut-off of 3.5 kDa. After elution, the sample was concentrated using a 1.5 ml microcentrifuge filtration unit with a molecular mass cut-off of 5 kDa (Ultrafree MC, Millipore Corp., Bedford,

USA). After washing twice with PBS in the same filtration unit, the sample was taken up in 50 μl PBS and stored at -20 °C. The thawed sample was used for intrasplenic immunization.

Immunizations and cell fusions

For the intraperitoneal immunizations (protocols 1 and 2) the antigen for the first two immunizations was mixed 1:1 with Freund's incomplete adjuvant, while the final booster immunizations were done without adjuvant. Intrasplenic immunizations (protocols 3 and 4) were given only once, and performed according to Spitz *et al.* (1984). In all cases the mice were sacrificed 3 days after the final immunization and MAb-producing hybridoma cell lines were obtained by fusing spleen cells with SP 2/0 myeloma cells (Goding, 1983; Schots *et al.*, 1992 *b*).

Immunofluorescence microscopy

Labelling of preparasitic J2 of *G. rostochiensis* for indirect immunofluorescence testing of MAbs was done essentially according to Atkinson *et al.* (1988) and Hussey (1989 *b*). The J2 were fixed in 2% paraformaldehyde in PBS for 2 or 3 days. The nematodes were then washed in distilled water and drops of concentrated suspension were spread evenly onto aluminium dishes (diameter 2 cm), which were glued to microscope slides for easy manipulation. The drops were allowed to dry at room temperature in a box with silica gel, after which the dishes with nematodes were stored dry at -20 °C until used. After thawing, the dried J2 were cut into small sections on their aluminium dish using a piece of razor blade attached to a plastic pipette tip. By cutting parallel lines in three different directions most of the nematodes were cut in 2 or more pieces. The nematode sections were taken up in 1 ml of PBS containing 1 mg/ml proteinase K (Merck, Darmstadt, Germany) and incubated for 20 min with agitation at room temperature. Using centrifugation in a swing-out rotor (2 min 2000 *g*) the nematodes were subsequently taken up in cold methanol (1 min; -20 °C) and cold acetone (2 min; -20 °C). After removal of the acetone, the nematode sections were resuspended in blocking buffer containing PBS, 10% horse serum, and 1 mM phenylmethylsulfonyl fluoride. Labelling of the J2 was done in 96-well filtration plates with a pore size of 0.45 μm (MultiScreen-HV, Millipore, Bedford, USA). To each well 20 μl of nematode suspension (containing approximately 200 sections) was added, followed by 80 μl of hybridoma culture supernatant. After incubation overnight in a moist atmosphere, the nematode sections were washed three times with PBS/0.1% Tween-20 by applying vacuum to the filtration plates, and they were next incubated in the dark for 2 h with FITC-conjugated rat-anti-mouse IgG (Jackson Immuno Research Laboratories Inc, West Grove, USA), diluted to 1

μg/ml in PBS containing 0.1% BSA and 0.1% Tween-20. After three washes with PBS/0.1% Tween-20, the nematode sections were taken up in 20 μl of distilled water, and transferred to 24-well microscope slides (Cel-Line Associates Inc., New Field, USA) precoated with 0.1% poly-L-lysine (2 μl/well). After drying the slides in the dark, 2 μl of anti-quenching agent (0.5 M sodium carbonate buffer pH 8.6 with 0.2 mM p-phenylene-diamine, mixed 1:1 with glycerol) was applied to each well and a large coverslip was fixed to the slide with dots of nail polish. Specimens were viewed with a 50× water immersion objective using a Leitz epifluorescence microscope with an L 2.1 or I filter block. The MAbs were scored for specific reactions with structures of the J2, and cell lines producing antibodies of interest were retained. Heavy and light chain isotyping of the MAbs was performed with hybridoma culture supernatants in a DAS ELISA (Schots *et al.*, 1992 *b*).

Immunofluorescence labelling of preparasitic J2 from *G. pallida, H. schachtii* and *M. hapla* with MAbs followed the same procedure as described for *G. rostochiensis*, with the exception that the initial fixations in paraformaldehyde were different: two days for *G. pallida*, and one day for *H. schachtii* and *M. hapla.* Immunofluorescence labelling of preparasitic J2 from *G. tabacum, H. glycines* and *M. incognita* was as described by Goverse *et al.* (1994).

ELISA and dot blots

Preparasitic J2 of *G. rostochiensis* were homogenized at 4 °C in 20 mM sodium phosphate buffer pH 8.0 using a small glass mortar and pestle, and the homogenate was stored at -80 °C until used. After thawing, the sample was centrifuged for 10 min at 10000 *g* and the supernatant was used. The ELISA was performed according to Schots *et al.* (1992 *b*) with the modification that the blocking buffer was PBS/0.1% Tween-20/0.5% BSA, and the incubation buffer was PBS/0.1% Tween-20/0.1% BSA. For testing of the supernatant fraction, the wells were coated with aliquots of supernatant equivalent to 20 J2, diluted in coating buffer. Assuming a total protein content of about 5 ng per J2 (De Boer *et al.*, 1992 *a*; Chapter 2) this corresponds to approximately 100 ng of protein/well. The MAbs were tested as hybridoma culture fluids diluted 1:10 in incubation buffer. For the dot blot assay of the supernatant fraction, aliquots of protein equivalent to 20 J2 were diluted in coating buffer and transferred to nitrocellulose membrane using a 96-well dot-blotting apparatus (Schleicher & Schuell, Dassel, Germany). Further labelling of the blots followed the same procedure as the ELISA, with the only difference that alkaline phosphatase activity was detected using nitro blue tetrazolium (see below).

SDS-PAGE and Western blotting

Mini SDS-PAGE was performed essentially as described (De Boer *et al.* 1992*b*; Chapter 3). Preparasitic J2 of *G. rostochiensis* were homogenized in 208 mM Tris-HCl pH 6.8 supplemented with 8.33% (v/v) 2-mercaptoethanol at 5 °C using a small glass mortar and pestle. Following homogenization the samples were mixed in a ratio of 3:2 (v:v) with a solution of 5% SDS/25% glycerol/0.1% Bromophenol Blue, thus producing standard SDS-sample buffer (O'Farrell, 1975). The homogenate was heated for 5 min in boiling water, centrifuged for 5 min at 10000 *g*, and the supernatant was stored at -80 °C until used. Per minigel approximately 10000 J2 were added to a single 73 mm wide slot in the stacking gel. An adjacent reference well (3 mm wide) was filled with prestained molecular weight markers (Bio-Rad, Hercules, USA). Following electrophoresis in a 13% acrylamide separating gel, the proteins were transferred to polyvinylidene difluoride membrane (Immobilon-P, Millipore Corp., Bedford, U.S.A) using a semi-dry electroblotting apparatus. A continuous transfer buffer system was used containing 39 mM glycine, 48 mM Tris and 20% (v/v) methanol. Transfer was carried out with 0.8 mA/cm^2 for 1 h. The blots were cut into strips, which were blocked overnight in PBS/0.1% Tween-20 (PBST) supplemented with 5% (w/v) defatted milk powder. Following a wash in PBST/1% milk powder, the strips were incubated for 2 h in hybridoma culture fluid, diluted 1:6 in PBST/1% milk powder. After washing three times in PBST/1% milk powder, the strips were next incubated individually in alkaline phosphatase conjugated rat-anti-mouse IgG (H+L) (Jackson Immuno Research Laboratories Inc, West Grove, USA) diluted 1:5000 in PBST/1% milk powder for 1 h. After washing in PBST/0.1% milk powder (1×) and PBST (3×), the strips were stained individually in 0.1 M ethanolamine-HCl pH 9.6, supplemented with 4 mM $MgCl_2$, 5-bromo-4-chloro-3-indolyl phosphate (0.06 mg/ml) and nitro blue tetrazolium (0.1 mg/ml).

Results

Immunofluorescence screening

The reactivities of the monoclonal antibodies which were obtained with the different immunization protocols are shown in Table 6.1. Four MAbs (MGR 14, 17, 19, 21) that bound specifically to the subventral oesophageal glands were obtained after immunization with a total protein homogenate of J2 (protocol 1). These MAbs reacted with the entire contents of the gland cells, including their extensions and their terminal ampullae (Fig. 6.1 A). While in the gland extensions it was sometimes possible to distinguish individual secretory granules, the labelling of the gland cell body was usually uniform. The subventral gland nucleus was not

Table 6.1. Immunofluorescence reaction of monoclonal antibodies raised against antigens from preparasitic second-stage juveniles (J2) of *Globodera rostochiensis*. The immunofluorescence cross-reactivity with J2 of other species of sedentary plant-parasitic nematodes is listed as "+" if labelling of identical structures was observed, and as "–" if not; (additional) reactions with other structures are indicated between brackets. The following immunizations were used: (1) homogenized whole J2, intraperitoneal immunization; (2) a crude pellet fraction from homogenized J2, intraperitoneal immunization; (3) anterior portions of J4 females, intrasplenic immunization; (4) a high molecular weight protein fraction from J2, intrasplenic immunization. The blank table entries were not determined.

Antibody	Isotype		Immuni-zation	Specificity of antibody	Immunofluorescence cross-reactivity					
	H	L		*Globodera rostochiensis*	*Globodera pallida*	*Globodera tabacum*	*Heterodera glycines*	*Heterodera schachtii*	*Meloidogyne hapla*	*Meloidogyne incognita*
MGR 14	IgG1	κ	1	Subventral oesophageal glands	+	+ (c)	– (c, l)	– (c, l)	–	– (c)
MGR 17	IgG1	κ	1	Subventral oesophageal glands	+	+ (l)	– (c, l)	– (c, l)	–	– (c)
MGR 19	IgG1	κ	1	Subventral oesophageal glands	+	+ (c)	–	–	–	–
MGR 21	IgG1	κ	1	Subventral oesophageal glands	+	+ (c)	– (c, l)	– (c, l)	–	– (c, l)
MGR 31	IgG1	κ	2	Subventral oesophageal glands	+	+	– (c, l)	– (l)	–	– (c)
MGR 33	IgM	κ	3	Intestinal lumen	– (gr)	– (ss)	– (ss, sk, l)	– (ss, sk, l)	– (gr)	–
MGR 37	IgM	κ	4	Intestinal lumen	– (gr)	– (c, m)	– (c, m)	– (c, m)	–	– (c, m)
MGR 3, 7, 20	IgG1	κ	1	Body-wall muscles (striated)	+			+	+	+
MGR 13, 16	IgG1	κ	1	Body-wall muscles (uniformly)	+			+	+	+
MGR 24, 25, 26	IgM	κ	2	Cuticle surface	+			–	–	–
MGR 29	IgM	κ	2	Cuticle surface	+			– (s)	–	–
MGR 18	IgG1	κ	1	Genital primordium	+			–	–	–
MGR 34	IgM	κ	3	Cell nuclei	+			+	+	+

c, cuticle; **gr**, fine granules; **l**, oesophageal lumen from stylet base through metacorporal pump chamber; **m**, fine muscles; **s**, fine specks; **ss**, stylet shaft; **sk**, stylet knobs

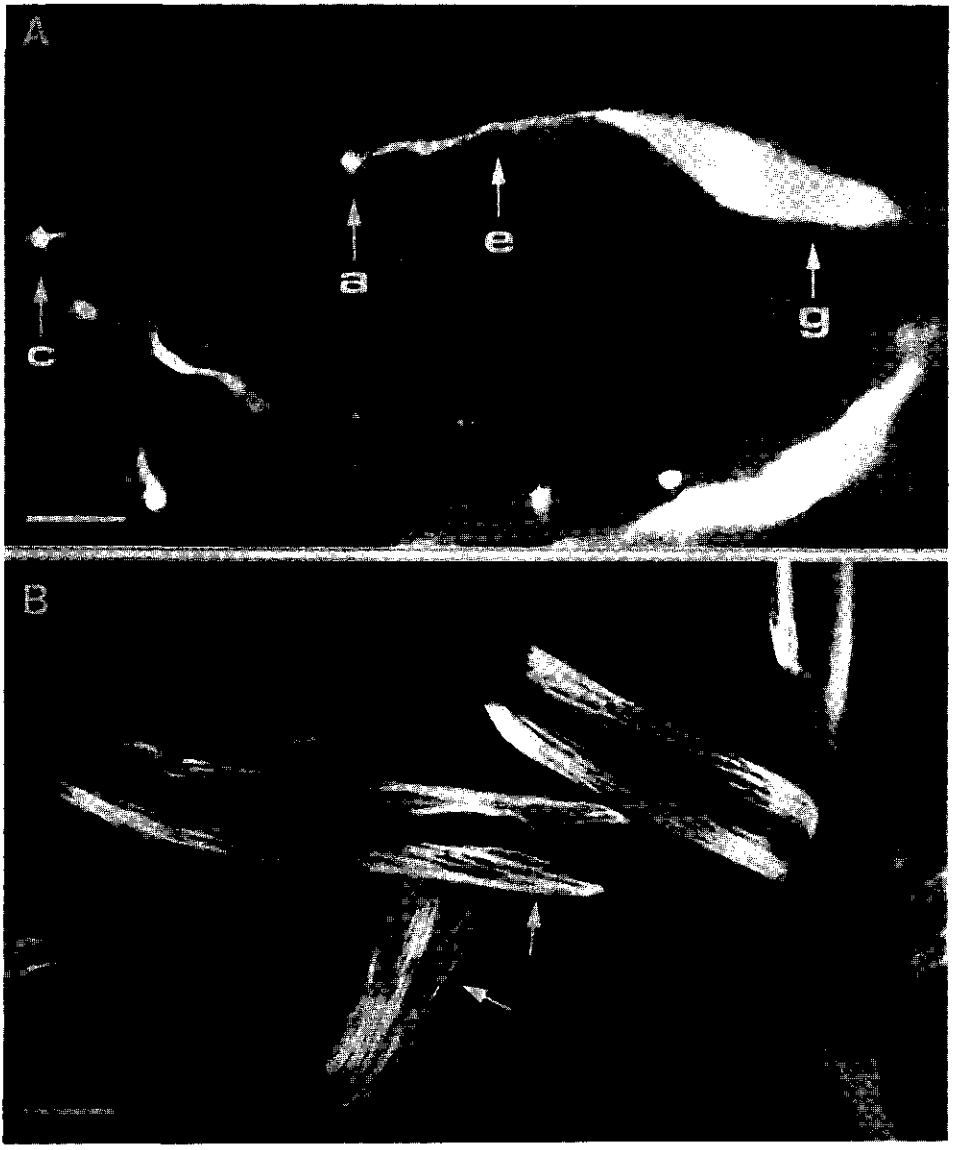

Fig. 6.1. Immunofluorescence labelling of second-stage juveniles (J2) of *Globodera rostochiensis* with monoclonal antibodies (MAbs). **A.** Labelling of the subventral oesophageal glands (g), their extensions (e) and ampullae (a) by MAb MGR 14. At the anterior end of the J2 the cephalic framework (c) is visible due to autofluorescence; **B.** Labelling of myofilaments in body-wall muscle cells by MAb MGR 7 reveals a fine pattern of oblique striations (arrows). Bars: 20 μm.

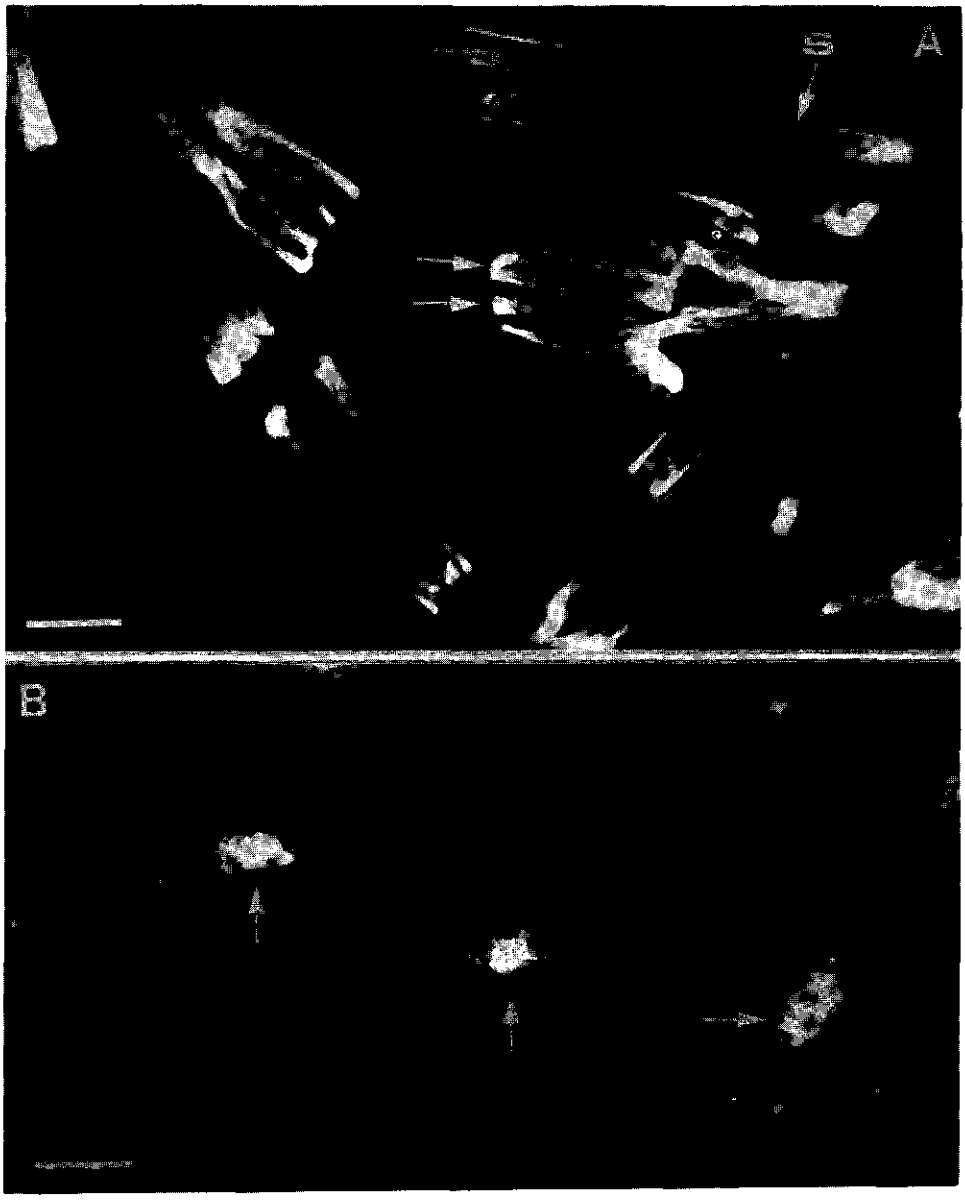

Fig. 6.2. Immunofluorescence labelling of J2 of *Globodera rostochiensis* with MAbs. **A.** Labelling of myofilaments in body-wall muscle cells (arrows) by MAb MGR 13. The myofilament lattice is usually stained more or less uniformly, and striations (s) are seen only occasionally in zones of less intense staining; **B.** Labelling of genital primordia (arrows) by MAb MGR 18. Bars: 20 μm.

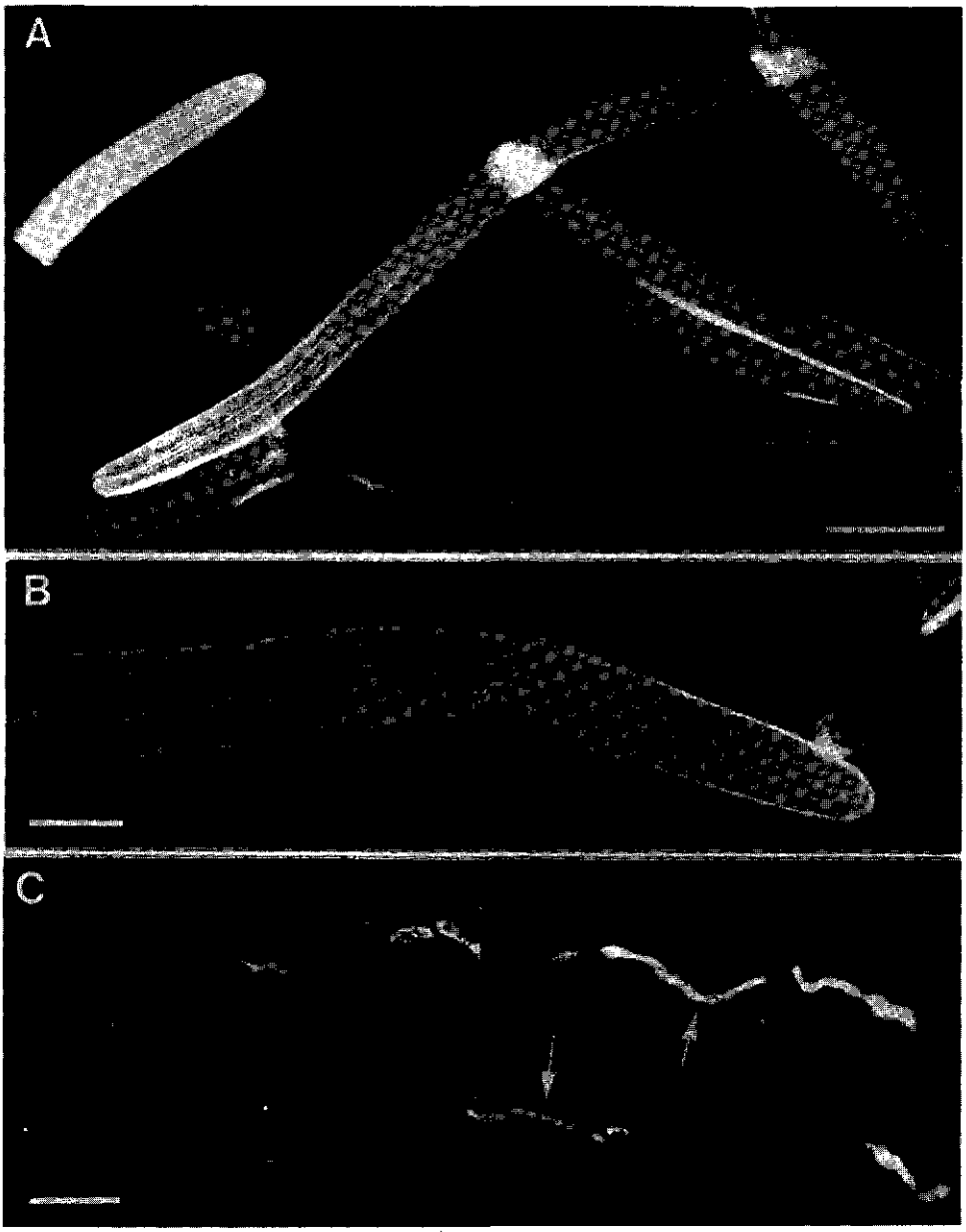

Fig. 6.3. Immunofluorescence labelling of J2 of *Globodera rostochiensis* with MAbs. **A.** Labelling of the cuticle surface by MAb MGR 29. Note the difference in labelling intensity between individual J2; **B.** Detail of cuticle surface labelling by MAb MGR 29. Both the cuticle annulations (a) and the lateral field lines (f) have become visible. **C.** Staining of the intestinal lumen (arrows) by MAb MGR 33. Bars: 50 μm (A) and 20 μm (B, C).

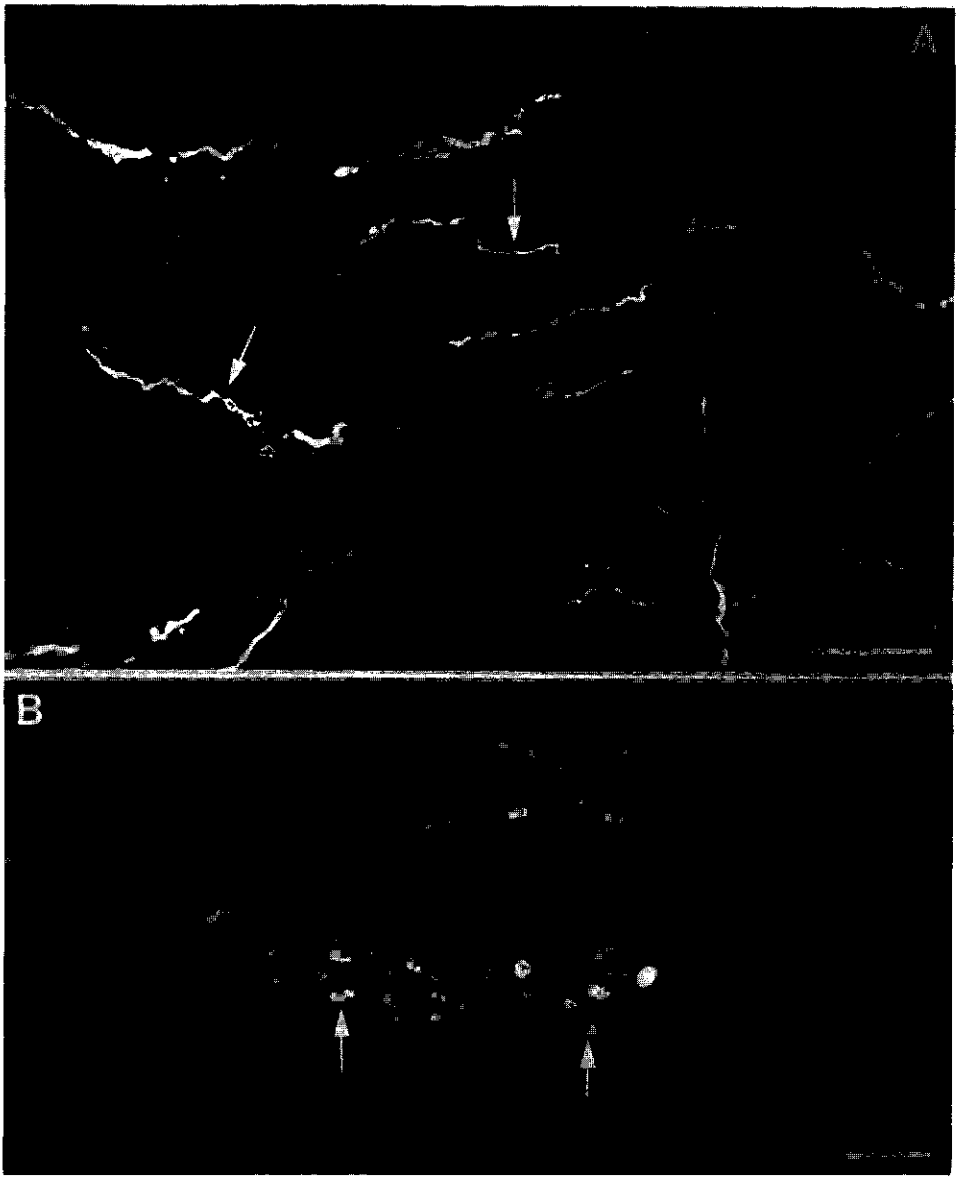

Fig. 6.4. Immunofluorescence labelling of J2 with MAbs. **A.** Staining of the intestinal lumen (arrows) by MAb MGR 33 in *Globodera rostochiensis*; **B.** Staining of cell nuclei (arrows) with MAb MGR 34 in *Heterodera schachtii*. The nucleoli remain unstained and are visible as a dark spots within the nuclei. Bars: 50 μm (A) and 20 μm (B).

labelled by these MAbs. Immunization protocol 1 had resulted in 47 MAbs to the body-wall myofilaments, and among these two types of binding patterns were observed. A few representative MAbs of each type were retained. Binding of MGR 3, 7 and 20 invariably followed a clear pattern of fine oblique striations within the muscle cells (Fig. 6.1 B). This differed from the binding pattern of MGR 13 and 16, which usually showed a more uniform labelling of the myofilament lattice (Fig. 6.2 A). With the latter MAbs, muscle striations could be seen only in zones of less intense labelling, and these striations appeared to be wider than those observed with MGR 3, 7, or 20. Finally, prototol 1 yielded a MAb (MGR 18) specific to the genital primordium (Fig. 6.2 B). Labelling predominated at the surface of the primordial cells and usually two large cells with two to four adjacent small cells could be discerned.

Immunization with a crude pellet fraction of J2 (protocol 2) produced one additional MAb against the subventral glands (MGR 31) which showed a staining pattern similar to the MAbs MGR 14, 17, 19 and 21 produced with protocol 1. From this immunization we also obtained four MAbs (MGR 24, 25, 26, 29) that bound to the cuticle surface (Fig. 6.3 A, B). Although these antibodies showed an even labelling of the entire cuticle surface, differences in staining intensity could be observed between individual J2.

Immunization with anterior portions of fourth stage females (protocol 3) and eluted high molecular weight proteins (protocol 4) yielded very few MAbs that showed specific labelling of structures within the J2. From these immunizations two MAbs were obtained (MGR 33 and 37) which stained a single thread-like structure in the centre of the J2, starting at the level of the subventral glands (presumably at the position of the oesophageal-intestinal valve) and ending in the tail (Figs. 6.3 C and 6.4 A). From this staining pattern it is concluded that these antibodies bind to the intestinal lumen. MGR 34 specifically labelled cell nuclei (Fig. 6.4 B), which were distributed over the entire length of the J2. Reaction with the large nuclei in the oesophageal glands or in the genital primordium was not observed with this antibody.

Cross-reactivity with other species

The MAbs raised against *G. rostochiensis* were tested with immunofluorescence microscopy for cross-reactivity with J2 of other sedentary plant-parasitic nematodes (Table 6.1). All five MAbs specific to the subventral glands in *G. rostochiensis* also reacted with epitopes in the subventral glands of *G. pallida* and *G. tabacum*. In *G. tabacum* the binding pattern was often granular as opposed to a uniform staining in *G. rostochiensis* and *G. pallida*. In the *Heterodera* and *Meloidogyne* species tested, no binding to the subventral glands was observed,

although reactions with the cuticle surface or the oesophageal lumen often occurred. The MAbs specific to the intestinal lumen of *G. rostochiensis* (MGR 33 and 37) did not react with the intestinal lumen in J2 from any of the other species. Instead, binding to various other structures occurred such as the stylet, the cuticle or the body-wall muscles. The MAbs that bound to the body-wall musculature and to cell nuclei bound to the same structures in the other species tested. MGR 24, 25, 26, and 29 all showed cross reactivity with the cuticle surface of *G. pallida* but did not bind to the cuticle surface of *H. schachtii, M. incognita* or *M. hapla*. MAb MGR 18 bound only weakly to the genital primordium in *G. pallida*, and did not bind to the genital primordia of the other species tested.

Western blotting

The MAbs presented in Table 6.1 were tested for reactivity with proteins from preparasitic J2 of *G. rostochiensis* which were separated by SDS-PAGE and blotted onto PVDF membrane. None of the MAbs to the subventral glands or to the intestinal lumen gave a positive reaction. Also MGR 18, 24, 25, 29 and 34 showed no reaction on Western blots. MGR 3 and 7 (Fig. 6.5) and MGR 20 (not shown) reacted with a major protein band with an apparent molecular mass of approximately 39 kDa. MGR 13 and 16 (Fig. 6.5) both reacted intensely with a protein band positioned above the 106 kDa molecular mass marker. In addition, MGR 13 stained several minor bands below 106 kDa. MGR 26 identified a series of protein bands with molecular masses of 39 kDa and more (Fig. 6.5).

ELISA and dot-blot

Because the subventral gland MAbs did not bind to SDS-denatured proteins on Western blots of *G. rostochiensis*, their reactivity with native protein homogenates from preparasitic J2 was tested using an ELISA and a dot-blot assay (Table 6.2). With three subventral gland MAbs (MGR 14, 17 and 31) antigen could now be detected in the protein homogenate. Control tests performed with four MAbs to body-wall muscle proteins (MGR 7, 13, 16, and 20) all gave positive reactions. Similar tests with MGR 18, 24, 25, 26, 29, 33, 34 and 37 (not in table) were negative, both in the ELISA and dot-blot assay. The subventral gland MAbs were tested also on dot blots of native homogenates of parasitic juveniles of *G. rostochiensis*. It was found that the reactivity of MGR 14, 17, and 31 had disappeared in the parasitic J2 stage, and did not reappear in later (J3, J4) parasitic stages. Control tests with muscle antibody MGR 7 remained positive in these parasitic stages.

Table 6.2. Reactivity of monoclonal antibodies with native protein homogenates from preparasitic J2 of *Globodera rostochiensis*; (+) positive reaction; (–) negative reaction.

Antibody	Specificity	ELISA	Dot-blot
MGR 14, 17	Subventral glands	–	+
MGR 31	Subventral glands	+	+
MGR 19, 21	Subventral glands	–	–
MGR 7, 13, 16, 20	Body-wall muscles	+	+

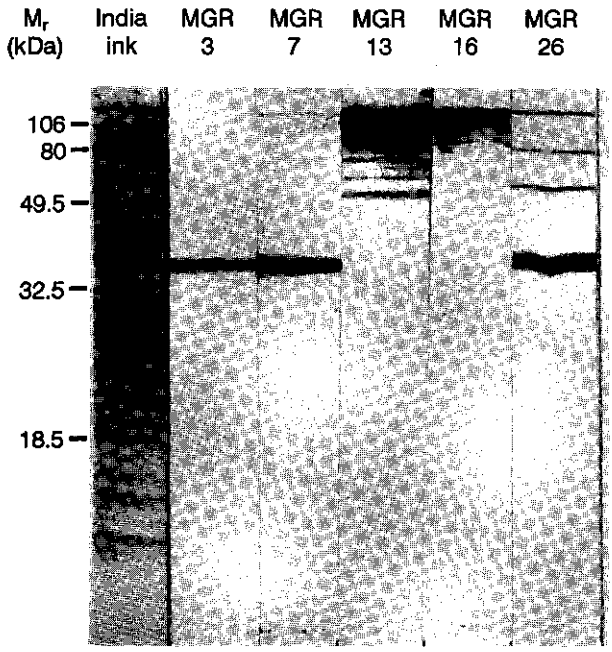

Fig. 6.5. Western blot of proteins from preparasitic J2 of *Globodera rostochiensis* stained with monoclonal antibodies to the body-wall muscle filaments (MGR 3, 7, 13 and 16) and to the cuticle surface (MGR 26).

Discussion

Following immunization with a total protein homogenate from J2 (protocol 1) MAbs were raised against the subventral oesophageal glands, the body-wall muscle fibres, and the genital primordium of *G. rostochiensis*. Similar antibodies were produced by Atkinson *et al.* (1988) when they immunized mice with a total protein homogenate from J2 of *H. glycines*. While Atkinson *et al.* (1988) also identified a MAb specific to the dorsal oesophageal gland following their immunization with homogenized J2, we did not observe this binding activity during the screening of the hybridomas.

Because the immunization with homogenized J2 had not produced MAbs specific to the dorsal oesophageal gland of *G. rostochiensis*, mice were also immunized with three samples that were expected to be enriched in antigens from the dorsal gland cell. The crude pellet fraction derived from J2 (protocol 2) was expected to contain secretory granules from both the dorsal and the subventral glands (Reddigari *et al.*, 1985). The intrasplenic immunization with anterior portions of female J4 (protocol 3) followed an immunization schedule which was successful in generating MAbs against the dorsal gland in *M. incognita* (Davis *et al.*, 1992). Finally, the high molecular weight protein sample eluted from an SDS-PAGE gel of J2 (protocol 4) was expected to contain high molecular weight secretory components of the oesophageal glands (Hussey *et al.*, 1990). However, none of these immunizations produced MAbs to the dorsal gland of *G. rostochiensis*. Because autofluorescence of the cuticle prevented immunofluorescence testing of antibodies with J4 females, the MAbs from protocol 3 were screened with J2. It is therefore possible that a difference in the expression of dorsal gland antigens between J4 females and J2 has prevented the detection of dorsal gland MAbs in this experiment.

The MAbs to the subventral oesophageal glands (MGR 14, 17, 19, 21 and 31) reacted with the entire contents of the gland cells, including the gland extensions to the median bulbus. This binding pattern is similar to that observed for MAbs to the subventral glands of J2 of *H. glycines* (Atkinson *et al.*, 1988; Goverse *et al.*, 1994). The MAbs raised by Goverse *et al.* (1994) bound specifically to secretory granules within the gland cell, and several of these MAbs were shown to react with induced stylet secretions released by J2. With our MAbs to the subventral glands of *G. rostochiensis* binding to individual secretory granules could sometimes be observed in the gland extensions. It is therefore possible that one or more of the subventral gland MAbs presented here react with a secretory product in the gland cells. In the cross-reactivity tests the subventral gland MAbs of *G. rostochiensis* only showed binding to the subventral glands of other *Globodera* species, and therefore the epitope that they recognize can be considered genus specific.

None of the subventral gland MAbs reacted with protein bands on Western blots of J2 of *G. rostochiensis*. Since MGR 14, 17 and 31 did react with supernatants of native protein extracts of J2, it can be concluded that their corresponding antigens are water soluble, and that their epitopes are susceptible to denaturation by SDS or mercaptoethanol. MGR 14, 17 and 31 did not bind to native protein homogenates of parasitic stages of *G. rostochiensis*. This may indicate that in these stages the subventral glands have switched to producing other secretory products (Atkinson & Harris, 1989; Endo, 1993; Davis *et al.*, 1994).

Two types of MAbs to the body-wall muscle filaments were identified. MGR 3, 7, and 20 stained thin oblique striations within the myofilament lattice (cf. Francis & Waterston, 1985), while MGR 13 and 16 showed a more even staining of the muscle filaments. This difference in immunofluorescence staining reaction corresponded with a different reaction on Western blots of J2: the former MAbs all bound to a 39 kDa protein, while the latter MAbs stained a major protein band with a molecular mass of > 106 kDa. These molecular mass values indicate that MGR 3, 7 and 20 may bind to tropomyosin and that the antigen identified by MGR 13 and 16 may be the myosin heavy chain subunit (De Boer *et al.*, 1992 *a*; Chapter 2). The staining of several additional thin protein bands by MGR 13 possibly reflects a susceptibility of this antigen to proteolysis during sample homogenization. An acute proteolytic susceptibility during homogenization has been reported for myosin of *Caenorhabditis elegans* (Harris & Epstein, 1977).

Genetic transformation with genes coding for antibodies (or fragments thereof) may offer a new route for introducing resistance to phytopathogens in plants (Benvenuto *et al.*, 1991; Schots *et al.*, 1992 *a*). Binding of *in planta* expressed antibodies to target molecules of phytopathogens can inhibit the function of these molecules and thus disturb the host-parasite interaction (Tavladoraki *et al.*, 1993). Suitable target molecules of endoparasitic nematodes are the secretions from the oesophageal glands (Hussey, 1989 *a*). The MAbs to the subventral glands of *G. rostochiensis* which have been presented here form a starting point for engineering resistance to cyst-nematodes in potato. They can be used for the purification and identification of antigens from the subventral gland cells. In addition, it may be possible that some of these MAbs are suitable for *in planta* inhibition of subventral gland secretions that are released by preparasitic J2 in the roots.

In conclusion, we have generated a panel of MAbs reacting with a variety of antigens of *G. rostochiensis*. These antibodies will be used in future molecular and structural studies concerning *G. rostochiensis* and its development in the host plant. Several of these MAbs showed cross-reactivity with other sedentary nematodes in immunofluorescence assays, and it can therefore be expected that these MAbs will be useful also for the study of similar antigens in these related nematode species.

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Chapter 7

Secretory granule proteins from the subventral oesophageal glands of the potato cyst-nematode identified by monoclonal antibodies to a protein fraction from second-stage juveniles

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Summary

Sodium dodecyl sulfate-extracted proteins from second-stage juveniles (J2) of the potato cyst-nematode *Globodera rostochiensis* were fractionated by preparative continuous flow electrophoresis, and monoclonal antibodies (MAbs) were raised against the 38- to 40.5 kDa protein fraction. Screening of the hybridoma culture fluids by immunofluorescence microscopy of J2 resulted in the identification of 12 MAbs that bound specifically to the subventral oesophageal glands. On Western blots of J2 these MAbs identified four protein bands with apparent molecular masses of 30, 31, 39 and 49 kDa. Immunoelectron microscopy with one of these MAbs showed an intense labelling of the electron dense core of the secretory granules in the subventral gland cells of J2. It is concluded that one or more of these proteins are localized within these secretory granules. Immunofluorescence microscopy of J2 from other plant-parasitic nematode species showed that most of these MAbs also bind to the subventral glands of *G. pallida* and *G. tabacum* but not of *Heterodera schachtii, H. glycines, Meloidogyne incognita,* or *M. hapla.*

Additional keywords: host-parasite interaction, virulence factor, host plant transformation, plantibodies.

Introduction

Potato cyst-nematodes are sedentary plant parasites that feed from their host by exploiting a syncytium of metabolically active root cells (Dropkin, 1969; Jones & Northcote, 1972; Jones, 1981; Melillo *et al.*, 1990). Their annual life cycle begins with hatching of second-stage juveniles (J2) from cysts in the soil. These preparasitic J2 penetrate the roots behind the growing tips, and following a short migration period within the root, each J2 selects a cortex cell for feeding site induction. The J2 insert its stylet into the cytoplasm of this cortex cell to induce its modification into an initial feeding cell, and after a short rest period the juvenile starts feeding (Steinbach, 1972; Steinbach, 1973; Wyss & Zunke, 1986; Wyss, 1992). At this stage the J2 has already transformed into a sedentary parasite, and the initial feeding cell soon expands to form an elaborate syncytium which transfers nutrients from the vascular tissue to the feeding nematode. After approximately 5 weeks of growth, the adult females have swollen to a globular shape, and they are filled with eggs. When they die, their tanned cuticle forms a protective cyst which harbours the diapausing J2 of the next generation (Evans & Stone, 1977).

Secretory products from the oesophageal glands of sedentary plant-parasitic nematodes are considered to play a major role in both induction and exploitation of the various types of feeding cells that these parasites establish in the roots of their host (Jones, 1981; Hussey, 1989*a*). The production of these salivary secretions takes place in two subventral gland cells and one dorsal gland cell (Endo, 1984; Hussey, 1989*a*). The salivary proteins are sequestered in secretory granules, which are transported into the narrow extension of the gland cell. Controlled release of the granule contents in the oesophagus occurs at the end of this extension. The subventral gland extensions terminate halfway along the oesophagus in the median pump chamber, whereas the dorsal gland extension ends just behind the stylet. It is very likely that some of the secretions from the dorsal gland are responsible for the formation of feeding tubes in the cytoplasm of the feeding cells (Rumpenhorst, 1984; Wyss & Zunke, 1986; Hussey & Mims, 1991; Wyss, 1992). The function of the subventral glands, however, is still unclear. It has been suggested that they release cell wall degrading enzymes during root invasion (Wyss *et al.*, 1992) or that they play a role in feeding cell induction (Atkinson *et al.*, 1988). Furthermore, it has been suggested that in parasitic stages subventral gland secretions move posteriorly towards the intestine for the mobilization of lipid reserves (Wyss, 1992; Wyss & Grundler, 1992), or for internal food digestion (Davis *et al.*, 1994). Immunofluorescence labelling of stylet secretions with monoclonal antibodies has demonstrated that in J2 and females of root-knot nematodes both gland types are capable of releasing their secretions via the stylet orifice (Hussey *et al.*, 1990; Davis *et al.*, 1994).

Monoclonal antibodies (MAbs) have been used in attempts to identify the oesophageal gland secretory proteins of the sedentary plant-parasitic nematodes *Heterodera glycines* (Atkinson *et al.*, 1988; Goverse *et al.*, 1994) and *Meloidogyne incognita* (Hussey, 1989*b*; Davis *et al.*, 1992). For both species, screening procedures using immunofluorescence microscopy have identified MAbs that react with secretory granules of the dorsal and subventral gland cells. Various antigens have been used to produce these monoclonal antibodies: with *H. glycines* mice were immunized with homogenates from hatched J2, unhatched J2, stylet secretions of J2, and adult females or anterior and posterior parts thereof (Atkinson *et al.*, 1988; Goverse *et al.*, 1994); with *M. incognita* the immunogens were a total protein homogenate of J2, a subcellular granule fraction from J2, anterior and posterior parts of females, and stylet secretions of females (Hussey, 1989*b*, Davis *et al.*, 1992). In both species the immunosuppressive drug cyclophosphamide was used to enhance the effect of the immunizations (Atkinson *et al.*, 1988; Davis *et al.*, 1992), and with *M. incognita* intrasplenic immunizations were employed to administer minute quantities of immunogen (Davis *et al.*, 1992). However, the success of these MAbs in identifying oesophageal gland secretory proteins, either by immunostaining of gel electrophoresis patterns or by purification with chromatography techniques, has remained limited. Hussey *et al.*, (1990) used a MAb reactive with both the dorsal and subventral glands in *M. incognita* to isolate a secretory component from homogenized J2. This protein had an apparent molecular mass of more than 212 kDa, and was glycosylated. A MAb binding to the subventral oesophageal glands of adult females of *M. incognita* was used to isolate a gene from a cDNA expression library (Ray *et al.*, 1994). Because this MAb also bound to the body-wall muscles in J2 of *M. incognita* and because the sequence of the isolated gene showed homology with the rod portions of myosin heavy chains, it was suggested that this antigen may be involved in the movement of secretory granules rather than being itself secreted (Ray *et al.*, 1994).

In this study, we report the production of MAbs that bind to secretory granule proteins from the subventral oesophageal glands of the potato cyst-nematode, *Globodera rostochiensis*. As antigen we used a protein fraction from homogenized preparasitic J2, which was collected by preparative continuous flow polyacrylamide gel electrophoresis. Hybridoma cell lines were screened by immunofluorescence microscopy for MAbs reacting with these oesophageal glands and these antibodies were subsequently used for identification of the secretory granule proteins on Western blots of J2. Electron microscopy was used to examine subventral gland morphology and to demonstrate the specificity of binding of one of these MAbs to the secretory granules within these glands. Finally, the MAbs were tested with

fluorescence microscopy for cross-reactivity with oesophageal glands in preparasitic J2 of other endoparasitic nematode species.

Materials and methods

Nematodes

Preparasitic second-stage juveniles (J2) of *Globodera rostochiensis* pathotype Ro_1 and of *G. pallida* pathotype Pa_2 were hatched by soaking cysts on a 100 μm sieve in potato root diffusate (Clarke & Perry, 1977). Preparasitic J2 of *Meloidogyne hapla* were a gift from Mrs. E. Jansen, DLO Research Institute for Plant Protection, Wageningen, The Netherlands. The J2 suspensions were mixed with an equal volume of 70% (w/v) sucrose in a centrifuge tube, covered with a layer of tap water, and centrifuged briefly at 1000 *g*. The purified J2 were collected from the sucrose-water interface with a Pasteur pipette, washed with tap water and used for experiments. Preparasitic J2 of *G. tabacum*, *Heterodera glycines*, *H. schachtii* and *M. incognita* were obtained as described by Goverse *et al.* (1994).

Preparative electrophoresis

In total 2.75 million J2 of *G. rostochiensis* were homogenized in 208 mM Tris-HCl pH 6.8 supplemented with 8.33% (v/v) 2-mercaptoethanol at 5 °C using a small glass mortar and pestle. The J2 were homogenized in aliquots of approximately 300,000 individuals. Following homogenization the samples were pooled and mixed in a ratio of 3:2 (v:v) with a solution of 5% SDS / 25% glycerol / 0.1% Bromophenol Blue, thus producing standard SDS-sample buffer (O'Farrell, 1975). The homogenate was heated for 5 min in boiling water, centrifuged for 5 min at 10,000 *g*, and the supernatant was stored at -80 °C until used. The nematode sample (2 ml, approximately 13 mg of protein) was fractionated by preparative SDS-PAGE (*cf.* Passmore *et al.*, 1992; Shain *et al.*, 1992; Fountoulakis *et al.*, 1993; Schnabl *et al.*, 1993) using a Model 491 Prep Cell apparatus (Bio-Rad, Hercules, California, USA) according to the instruction manual, with the correction that 0.1% SDS was added to the buffers of the acrylamide gels. The cylindrical separating gel was 50 × 28 mm (height × diameter) and contained 10% acrylamide. The stacking gel contained 4% acrylamide and was 15 mm in height. The proteins were separated with a current of 40 mA, and 1.5 ml fractions were collected using an elution buffer flow rate of 1 ml/min. The fractions were concentrated by freeze drying, washed three times in phosphate buffered saline pH 7.4 (PBS) using 1.5 ml microcentrifuge filter concentration units with a molecular mass cutoff of 5 kDa (Ultrafree-MC; Millipore Corp., Bedford, USA), taken up in 50 μl PBS and stored at -20 °C. Groups of 2 to 5 fractions were

pooled, and each pooled sample was diluted in PBS to obtain three 200 μl aliquots for successive immunizations.

Immunizations and cell fusions

Sixteen mice were immunized intraperitoneally with the isolated protein fractions, which were mixed 1:1 with Freund's incomplete adjuvant. After 4 weeks a second immunization was given, also with incomplete adjuvant. Two weeks later antiserum samples were collected for immunofluorescence microscopy. One mouse that was selected for monoclonal antibody production received a final booster injection 12 weeks after the first immunization. Three days later the mouse was sacrificed, and MAb-producing hybridoma cell lines were obtained by fusing spleen cells with SP 2/0 myeloma cells (Goding, 1983; Schots *et al.*, 1992 *b*).

Immunofluorescence microscopy

Mouse antisera and hybridoma cell line culture fluids were screened by immunofluorescence microscopy for reaction with preparasitic J2 of *G. rostochiensis* as described in detail elsewhere (J.M. de Boer *et al.*, 1996; Chapter 6). Briefly, J2 were fixed in 2% paraformaldehyde for 3 days, dried, and cut into sections with a razor blade. Following treatment with proteinase-K, methanol, and acetone, the nematode sections were incubated with the primary antibody and stained with rat-anti-mouse FITC-conjugated immunoglobulins. The mouse antisera were tested in a 1:400 dilution. Hybridoma cell lines producing antibodies to the subventral oesophageal glands of *G. rostochiensis* were subcloned to stability and stored in liquid nitrogen. Isotyping of the light and heavy chains of the MAbs was as described by Schots *et al.* (1992 *b*). Immunofluorescence testing of preparasitic J2 from *G. pallida* and *M. hapla* followed the same procedure as for *G. rostochiensis*, with the exception that the initial fixations in paraformaldehyde were different: two days for *G. pallida*, and one day for *M. hapla*. Immunofluorescence testing of preparasitic J2 from *G. tabacum*, *M. incognita*, *H. glycines* and *H. schachtii* was as described by Goverse *et al.* (1994).

SDS-PAGE and Western blotting

Analytical mini SDS-PAGE was performed essentially as described by De Boer *et al.* (1992) (Chapter 3). For the examination of the protein fractions that were obtained with preparative electrophoresis, 4 μl of SDS-sample buffer was added to 2 μl from the concentrated fractions in PBS, and these samples were applied to 20 μl slots in the stacking gel. Following electrophoresis in a 13% separating gel, the gels were stained with colloidal Coomassie Blue G-250 (Neuhoff *et al.*, 1988).

For Western blot testing of MAbs, J2 of *G. rostochiensis* were homogenized as described above, and per minigel approximately 10000 J2 were added to a single 73 mm wide slot in the stacking gel. An adjacent reference well (3 mm wide) was filled with prestained molecular weight markers (Bio-Rad, Hercules, USA). Following electrophoresis the proteins were transferred to polyvinylidene difluoride membrane (Immobilon-P, Millipore Corp., Bedford, USA) using a semi-dry blotting apparatus. A continuous transfer buffer system was used containing 39 mM glycine, 48 mM Tris and 20% (v/v) methanol. Transfer was carried out with 0.8 mA/cm^2 for 1 h. The blots were cut into strips, which were blocked overnight in PBS pH 7.4 / 0.1% (v/v) Tween-20 (PBST) supplemented with 5% (w/v) defatted milk powder. Following a wash in PBST / 1% milk powder, the strips were incubated for 2 h in hybridoma culture fluid, diluted 1:6 in PBST/1% milk powder. For MAb MGR 48 affinity-purified antibody (1 μg/ml) was used instead of culture fluid. After washing three times in PBST / 1% milk powder, the strips were next incubated individually in alkaline phosphatase conjugated rat-anti-mouse IgG (H+L) (Jackson Immuno Research Laboratories Inc, West Grove, USA) diluted 1:5000 in PBST / 1% milk powder for 1 h. After washing in PBST / 0.1% milk powder (1×) and PBST (3×) the strips were stained individually in 0.1 M ethanolamine-HCl pH 9.6, supplemented with 4 mM $MgCl_2$, 0.06 mg/ml 5-bromo-4-chloro-3-indolyl phosphate and 0.1 mg/ml nitro blue tetrazolium.

Electron microscopy

For ultrastructural examination, J2 of *G. rostochiensis* were fixed at 4 °C with 4% paraformaldehyde in 0.1 M HEPES-buffer pH 7.5 for 2 days. The suspension of fixed J2 was spread on a microscope slide and the nematodes were chopped into pieces with a razor blade. The nematode sections were then sequentially fixed with 2% glutaraldehyde and 1% osmium tetroxide (both in 0.1 M HEPES pH 7.5) and stained with 1% aqueous uranyl acetate. Following stepwise dehydration in ethanol, the J2 were infiltrated for 1 day with Spurr epoxy resin (Spurr, 1969). The nematode fragments were then transferred to fresh epoxy resin in a BEEM capsule, centrifuged to the bottom, and polymerized at 60 °C. Thin sections were cut with an LKB ultra-microtome, collected on formvar-coated 100 mesh copper grids, and poststained with 2% uranyl acetate in 50% methanol.

For immunolabelling, J2 of *G. rostochiensis* were fixed for 2 days at 4 °C in 2% paraformaldehyde in PBS pH 7.4. After chopping them into pieces, the J2 were washed 2 times in distilled water, dehydrated in 30%-50%-70%-96% ethanol, infiltrated at room temperature for 1 h in a 1:1 mixture of 96% ethanol and LR-White acrylic resin (London Resin Co. Ltd., Basingstoke, England), and subsequently in pure LR-White resin for 4 h. The nematode fragments were

transferred to fresh resin in a gelatin capsule, centrifuged to the bottom, and polymerized at 60 °C. Following ultramicrotomy, thin sections were collected on formvar-coated copper grids and immunolabelled with colloidal gold according to the following protocol (Aurion Immuno Gold Reagents, Wageningen, The Netherlands): 10 min PBS pH 7.6 / 0.05 M glycine, 2 h affinity purified MAb MGR 48 diluted to 1 μg/ml in incubation buffer (PBS pH 7.6 / 0.2% BSA-C / 20 mM NaN_3), 6× 5 min wash with incubation buffer, 2 h colloidal gold solution (10 nm particle size, conjugated with goat-anti-mouse-IgG immunoglobulins; Aurion Immuno Gold Reagents, Wageningen, The Netherlands) diluted 1:10 in incubation buffer, 6× 5 min wash with incubation buffer, 3× wash with PBS, and 3× wash with distilled water. The sections were stained with 1% aqueous uranyl acetate. Control labelling experiments were performed with a mouse monoclonal antibody specific to the body-wall musculature of J2 of *G. rostochiensis*.

Results

Antibody production

Homogenized J2 of *G. rostochiensis* were separated by preparative continuous flow electrophoresis in the presence of sodium dodecyl sulfate (SDS) into 50 protein fractions. The average molecular mass of these proteins fractions ranged from 30 to 52 kDa. Examination of successive fractions by analytical SDS polyacrylamide gel electrophoresis (SDS-PAGE) revealed narrow protein zones, with a very gradual increase in molecular mass (Fig. 7.1). Therefore the original fractions were pooled to form a representative set of 16 protein samples of increasing molecular mass, which were used for immunizing 16 mice. Antisera collected after the second immunization with these samples were screened with immunofluorescence microscopy for reaction with the oesophageal glands of J2 of *G. rostochiensis*. It was found that the eight mice that were immunized with the successive protein fractions from 36.5 to 52 kDa had produced antisera that showed a strong reaction with the subventral oesophageal glands (Fig. 7.2 A). In addition, a very weak reaction with the subventral glands was observed with the antiserum to the 31- to 33-kDa fraction. The mouse that was immunized with the 38- to 40.5-kDa protein sample (Fig. 7.1) was chosen for monoclonal antibody production.

Immunofluorescence microscopy

Screening of the hybridoma culture supernatants by immunofluorescence microscopy of nematode sections identified 12 MAbs that bound specifically to the subventral glands of J2 of *G. rostochiensis* (Fig. 7.2 B-C-D). The staining of the glands by the MAbs was found to be variable, both between individual J2

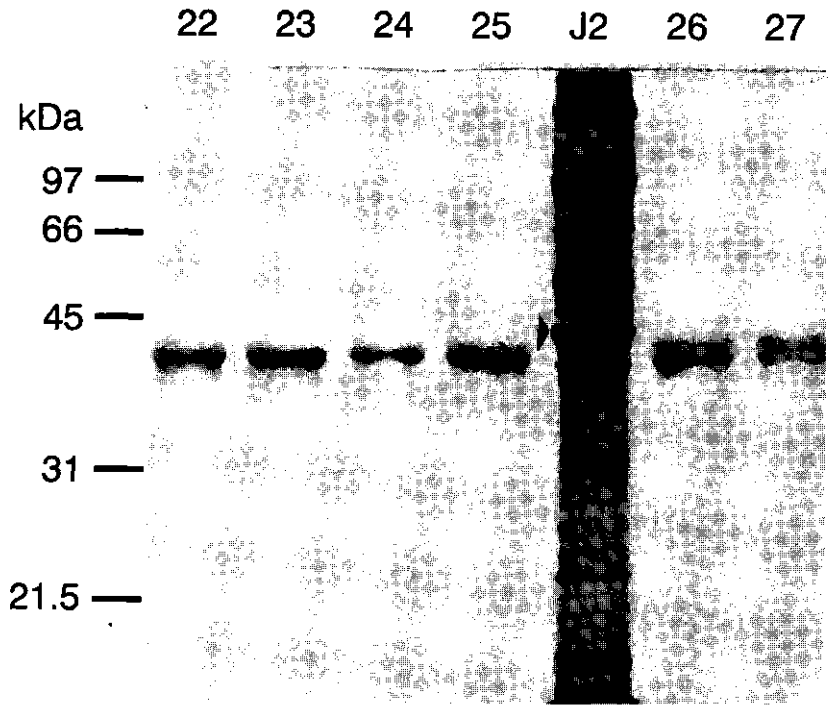
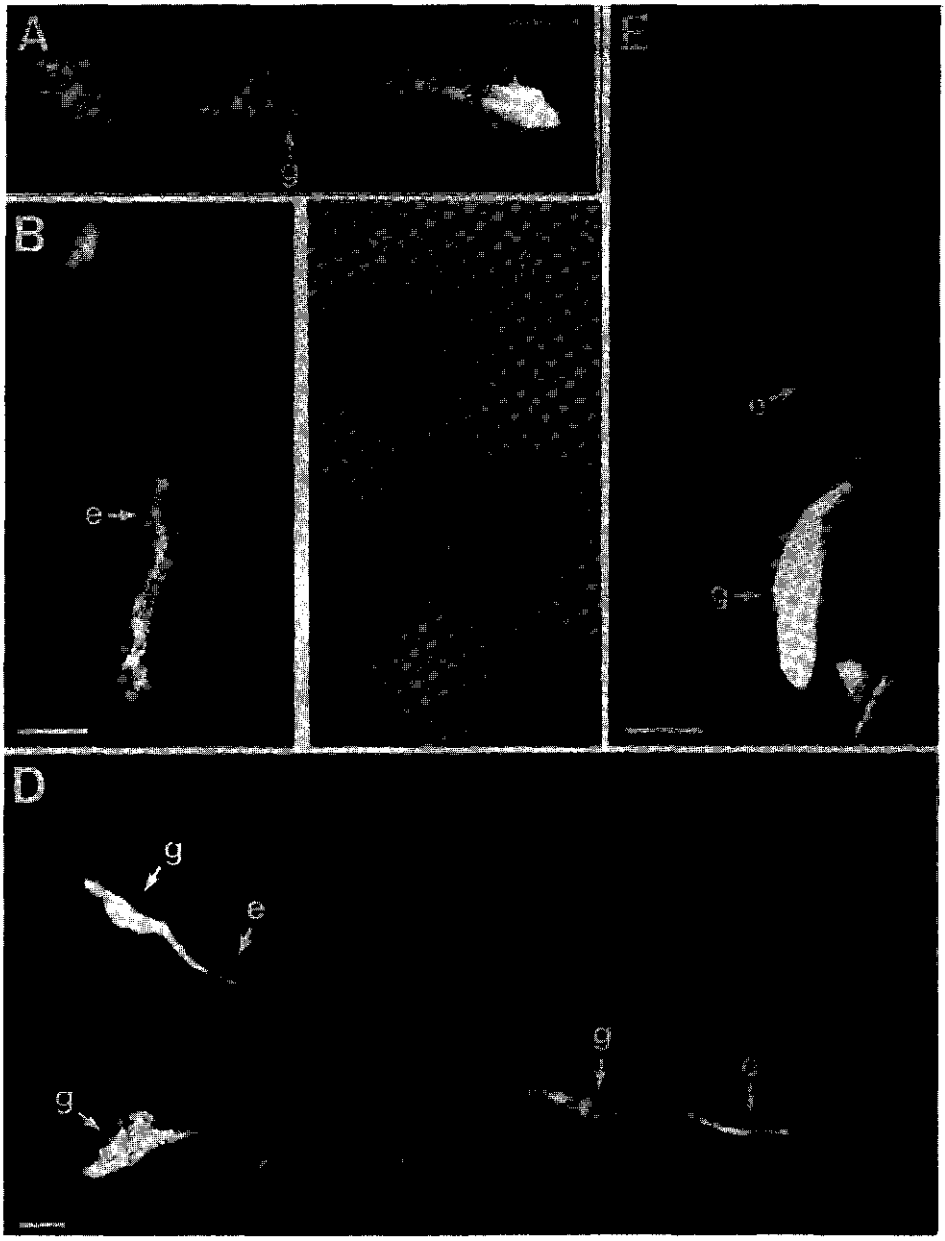


Fig. 7.1. SDS-polyacrylamide gel electrophoresis pattern of selected protein fractions purified from homogenized preparasitic second-stage juveniles (J2) of *Globodera rostochiensis* by preparative continuous flow electrophoresis. The lanes numbered 22 to 27 show successive fractions of increasing molecular weight. The mouse that was immunized with the pooled fractions 22 to 25 (molecular mass range 38 to 40.5 kDa) produced antiserum to the subventral oesophageal glands, and was used for monoclonal antibody production. The lane labelled "J2" shows a total protein homogenate of J2. The arrowhead indicates a characteristic major protein band (presumably actin) which can be used to compare Fig. 7.1 with Fig. 7.3.

Fig. 7.2 (opposite page). Immunofluorescence labelling of the subventral oesophageal glands (g) and their extensions (e) in preparasitic J2 of potato cyst-nematodes. (Because both glands lie in close apposition, their cell bodies and their extensions appear as single structures in the micrographs). **A.** *Globodera rostochiensis* stained with mouse antiserum raised to a 38.5- to 40-kDa protein fraction derived from J2 of *G. rostochiensis.* Individual secretory granules can be distinguished within the gland cells. **B, C, and D.** *G. rostochiensis* labelled with monoclonal antibody MGR 48. Additional bright field illumination (C) shows that the gland extensions terminate in the metacorpus (m) of the oesophagus. **E.** *G. pallida* labelled with monoclonal antibody MGR 48. (Due to the cutting of the J2 prior to immunolabelling, the subventral glands have come to lie outside this juvenile, with only their extensions still in place). Bars: 20 μm.


A
g
B
e
C
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D
g
e
g
g

Table 7.1. List of monoclonal antibodies to the subventral oesophageal glands of preparasitic J2 of *Globodera rostochiensis* and their immunofluorescence cross-reactivity with preparasitic J2 of other species of sedentary plant-parasitic nematodes. "+" reaction with subventral glands, "–" no reaction with subventral glands.

Antibody[a]	Isotype Heavy	Light	*Globodera pallida*	*Globodera tabacum*	*Heterodera schachtii*	*Heterodera glycines*	*Meloidogyne incognita*	*Meloidogyne hapla*
MGR 46	IgG2a	λ	+	+	–	–	–	–
MGR 47, 50, 53, 55, 56, 59	IgG1	κ	+	+	–	–	–	–
MGR 48	IgG1	κ	+	+[b]	–	+	–	–[b]
MGR 49	IgG3	κ	+	–	–	–	–	–
MGR 54	IgG2a	κ	+	+	–	–	–	–
MGR 57, 60	IgG1	λ	+	+	–	–	–	–

[a] Affinity-purified antibody (MGR 48) or hybridoma culture fluid (all other MAbs)
[b] Background staining

within a single experiment and between repeated experiments with the same antibody. Nevertheless, 10 MAbs (MGR 46, 47, 48, 49, 50, 53, 54, 55, 56, 59) were able to stain the entire contents of subventral gland cells, including the gland extensions (Fig. 7.2 D). Two MAbs (MGR 57 and 60) persistently failed to penetrate the gland cells sufficiently to obtain an even staining reaction.

Immunofluorescence microscopy was also used to test the cross-reactivity of the MAbs with J2 of other species of plant-parasitic nematodes (Table 7.1). It was found that nearly all MAbs also bound to the subventral glands of *G. pallida* (Fig. 7.2 E) and *G. tabacum*. However, except for MGR 48, which reacted with *H. glycines*, none of the MAbs showed binding to the oesophageal glands of the other species examined.

Western blotting

The MAbs were tested for reactivity with protein homogenates of J2 which were separated by SDS-PAGE and electroblotted onto PVDF membrane. Eleven of the 12 MAbs stained protein bands in the electrophoresis pattern (Fig. 7.3). Together these MAbs identified 4 different polypeptides with apparent molecular masses of 30 kDa (svp30), 31 kDa (svp31), 39 kDa (svp39) and 49 kDa (svp49) respectively. All reactive MAbs bound to the svp39 band, with 10 showing additional binding to one or more of the other bands. MGR 47, 53, 54, 55, and 59 all reacted strongly with the svp30, svp31 and svp39 bands, while MGR 46, 57 and 60 showed moderate binding to svp39 and svp49. Reactivity with all four

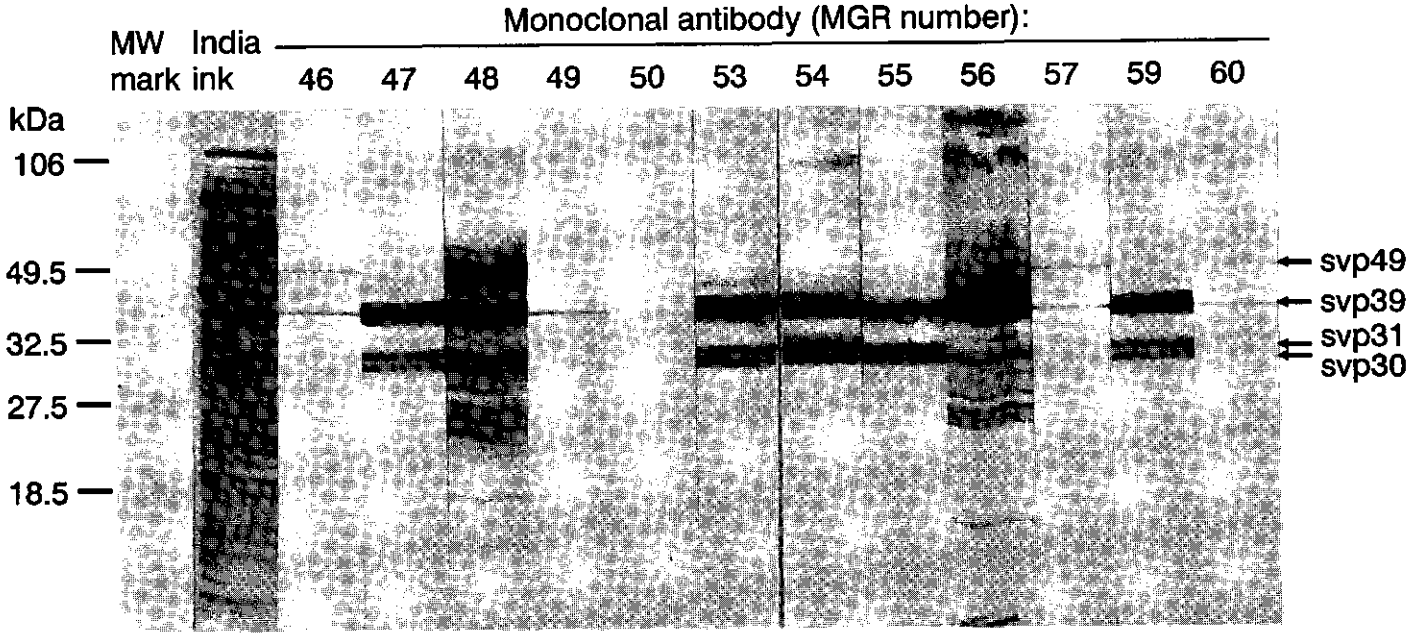

Fig. 7.3. Western blot of preparasitic second-stage juveniles (J2) of *Globodera rostochiensis* stained with a panel of monoclonal antibodies (MGR 46 to 60) specific to the subventral oesophageal glands. Four major protein bands are identified, labelled svp30, svp31, svp39, and svp49. MW Mark = prestained molecular weight markers. India ink staining shows the complete protein pattern of J2. The arrowhead indicates a characteristic major protein band (presumably actin) which can be used to compare Fig. 7.3 with Fig. 7.1.

protein bands was found only with MGR 48. In addition, MGR 48 stained several minor bands in the gel region below svp30. Staining of minor bands was also observed with MGR 56, both below svp30 and above svp39.

Electron microscopy

Because ultrastructural information on the subventral glands in potato cyst-nematodes was completely lacking and could only be inferred from studies with related plant-parasitic nematode species (Endo, 1984; Endo, 1988; Endo, 1993), we have first examined subventral gland morphology in preparasitic J2 of *G. rostochiensis*. Thin sections showed that both the cell body (Fig. 7.4) and the cell extension are packed with secretory granules. It was only in the immediate

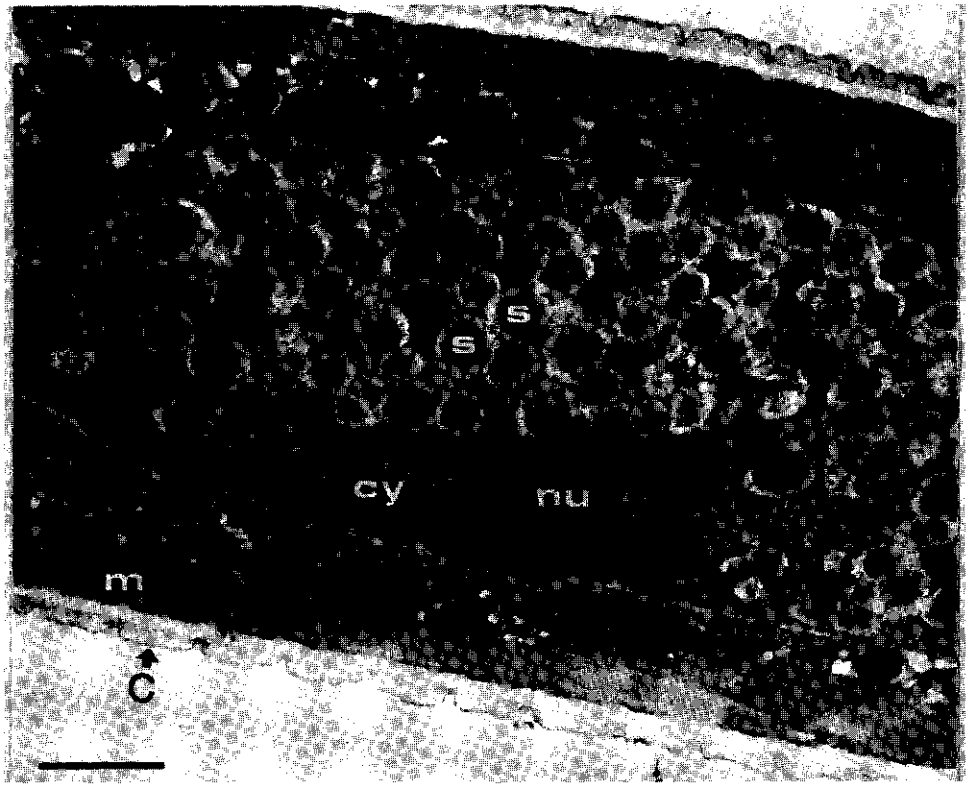

Fig. 7.4. Longitudinal section through the subventral gland region of a preparasitic second-stage juvenile of *Globodera rostochiensis*. The two subventral gland cells (g1 and g2) lie adjacent to each other, and they are both packed with secretory granules (s). Cytoplasm free of secretory granules (cy) could only be found near the subventral gland nucleus (nu). m = body wall muscle filaments. C = cuticle. Bar = 2 μm.

vicinity of the nucleus that parts of the cytoplasm were free of granules. The secretory granules invariably possessed an electron translucent halo which surrounded an electron dense core. This morphology made it easy to identify the subventral glands in the low-contrast formaldehyde fixed specimens that were used for immunolabelling.

Immunogold labelling of formaldehyde-fixed J2 with MAb MGR 48 was localized to the subventral oesophageal glands. Binding of the gold particles occurred both to the electron dense granule matrix and to the patches of cytoplasm surrounding the granules (Fig. 7.5).

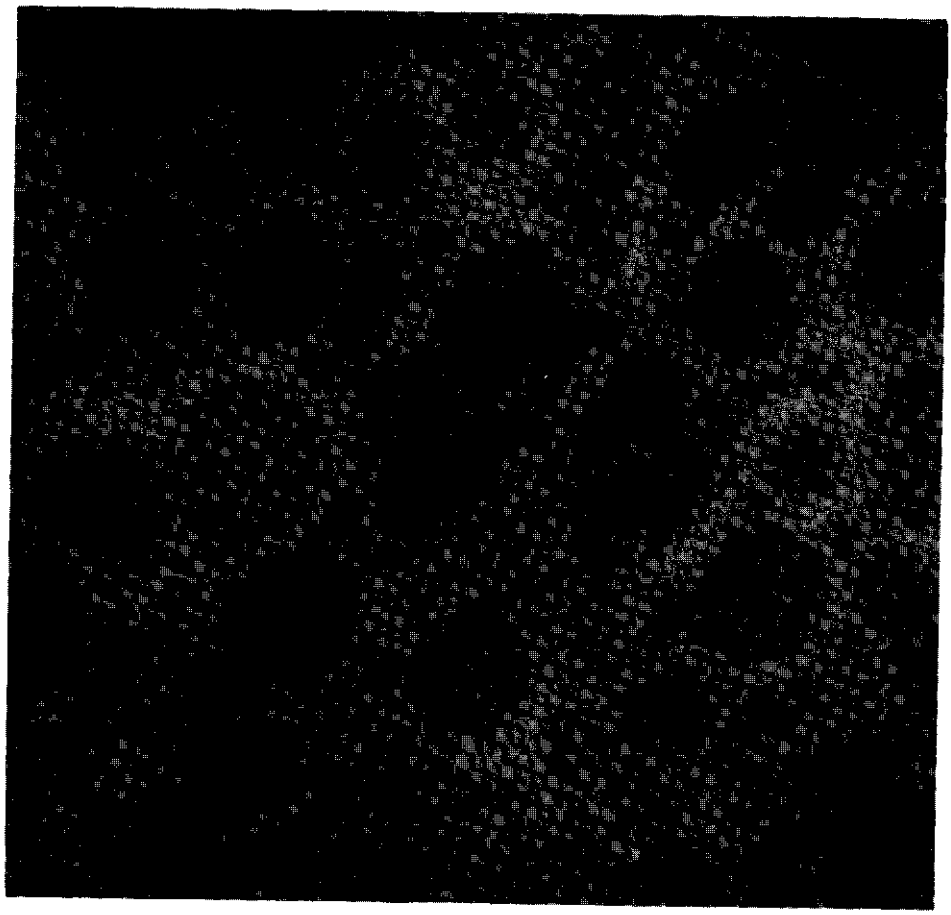

Fig. 7.5. Immunogold labelling of a subventral oesophageal gland of a preparasitic J2 of *Globodera rostochiensis* with monoclonal antibody MGR 48. Fixation with formaldehyde, and embedding in LR-White resin. The gold particles localize to the relatively electron dense core of the secretory granules (S). Labelling is also found outside the granules (e.g. arrows). Bar = 0.5 μm.

Discussion

In this paper we used a novel method of immunogen preparation to produce monoclonal antibodies specific to oesophageal gland antigens of a sedentary plant-parasitic nematode. Mice were immunized with protein fractions which were produced by preparative continuous flow SDS-electrophoresis of homogenized J2. Following immunofluorescence screening of hybridomas we identified a panel of 12 MAbs specific to the subventral glands of J2 of *G. rostochiensis*. Most of these antibodies differed from MAbs which we obtained previously to the subventral glands of *G. rostochiensis* (J.M. de Boer *et al.*, 1996; Chapter 6) in that they bound to SDS-denatured epitopes. This enabled the identification of subventral gland specific proteins on Western blots of J2 of *G. rostochiensis*.

The panel of MAbs presented here identified four major protein bands in the electrophoresis pattern of J2 of *G. rostochiensis*. All the MAbs that gave a positive reaction on Western blot identified a protein band of 39 kDa (svp39). This molecular mass corresponds with the protein fraction that was used for immunization. In addition three other proteins with smaller and larger molecular masses (svp31, svp32, and svp 49) were identified. It is likely that these additional polypeptides are structurally related to the svp39 band. Possibly these four proteins represent variants of a single gene product, which differ in post-translational modification or in the length of the polypeptide chain. The different reactivities with our panel of MAbs would then give information about the epitopes that are available on these related polypeptides. Thus MGR 47, 53, 54, 55, and 59 may be directed to an epitope that is present only on the svp30, svp31 and svp39 bands, whereas MGR 46, 57 and 60 may bind to an epitope specific for svp39 and svp49.

Monoclonal antibody MGR 48, which showed a reaction with all four protein bands on Western blots of J2, was used to determine the ultrastructural location of these polypeptides within the subventral gland. Immunogold labelling showed an intense binding of MGR 48 to the electron dense core of the secretory granules. It is therefore concluded that one or more of the proteins identified by MGR 48 are localized within the secretory granules of the subventral gland. Some labelling was also found in patches of cytoplasm between the granules. This could be the result of a release of antigen from the secretory granules due to the combined effects of mild tissue fixation and ethanol dehydration during specimen preparation. Alternatively, this may indicate that one or more of the polypeptides (also) reside outside the granules, for instance in the endoplasmic reticulum.

Preliminary *in vitro* experiments have demonstrated that the proteins identified by MAb MGR 48 can be retrieved in stylet exudates of preparasitic J2 of *G. rostochiensis* (G. Smant *et al.*, unpublished). It is therefore likely that the anti-

gens recognized by MGR 48 are secretory products of the subventral oesophageal glands. Their release via the stylet indicates that these proteins may play a role in the initial stages of root infection by J2. In that case they could be cell wall degrading enzymes that assist in root penetration (Steinbach, 1972) or signal molecules involved in feeding site induction (Steinbach, 1973).

The possibility of expressing genes coding for monoclonal antibodies in plants (Hiatt *et al.*, 1989) has raised the idea that antibodies may be suitable for introducing resistance to phytopathogens into crops (Benvenuto *et al.*, 1991; Schots *et al.*, 1992*a*). Binding of antibody to antigen by itself may exert an inhibiting effect on the antigen (Schots *et al.*, 1992*a*), and by choosing suitable target proteins in a plant pathogen, monoclonal antibodies ('plantibodies') to these targets could interfere with the host-parasite interaction. An example of plant pathogen inhibition by antibodies has recently been obtained for artichoke mottled crinkle virus infection in transgenic *Nicotiana benthamiana* (Tavladoraki *et al.*, 1993). Also in vertebrate cells this principle of *in situ* blockage of a biological function by intracellular expression of antibodies has been demonstrated (Biocca *et al.*, 1994; Werge *et al.*, 1994). *In planta* expression of antibodies to oesophageal gland secretions may offer new possibilities for introducing resistance to sedentary nematodes in plants. By inhibiting the function of these secretions, either following their release in the apoplasmic space, or following their injection into the cytoplasm of the host cell, the juvenile nematodes may be deprived of their food source and thus be arrested in their growth and development. The MAbs to subventral gland antigens of *G. rostochiensis* which have been presented here may prove to be useful for the control cyst-nematodes in potato. Transformation experiments are under way in our laboratories to test this hypothesis.

In conclusion, we have identified subventral gland specific proteins of preparasitic J2 of *G. rostochiensis* using monoclonal antibodies which were raised against a highly purified SDS-denatured immunogen. These antibodies will be valuable tools in future studies relating to the nature of these antigens and their possible function in the host-parasite interaction. We have shown that immunization with electrofractionated total protein homogenates can be a useful method for obtaining MAbs against oesophageal gland antigens of plant-parasitic nematodes. It is expected that by using fractionated proteins from other molecular weight ranges or from other developmental stages, a systematic search for additional oesophageal gland antigens of *G. rostochiensis* will be possible.

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Summary

Secretory proteins from the dorsal and subventral oesophageal glands of potato cyst-nematodes (*Globodera rostochiensis* and *G. pallida*) are considered to play an important role in the induction and exploitation of the specialized feeding cells (syncytia) that these parasites establish in the roots of their host plant. Identification of these secretory proteins will provide insight into the mechanisms involved in the formation of these syncytia, and is likely to aid in the development of novel forms of (artificial) host plant resistance to potato cyst-nematode infection. This thesis presents the data which were collected during a search for the oesophageal gland secretory proteins of *G. rostochiensis*.

In the first part of the investigations (Chapters 2 to 5) the proteins of *G. rostochiensis* are examined using two-dimensional gel electrophoresis (2D-GE) followed by silver staining. Chapter 2 compares the protein patterns of two motile (2nd-stage juveniles (J2) and males) and two sedentary (4th-stage females and adult females) developmental stages using conventional 2D-GE in large gels. This demonstrated very large differences in protein composition between these four developmental stages, and it was estimated that 74% of the polypeptides visible in these gels showed variations in their presence or abundance. The highest similarity index values were calculated for the protein patterns of both motile stages (0.674) and of both sedentary stages (0.767) with decreased values for all other pairwise comparisons.

Chapter 3 introduces a miniature 2D-GE technique of which the high sensitivity is demonstrated by its ability to genotype single females of *G. pallida*. In Chapter 4 this mini 2D-GE technique is used to analyse the proteins of the complete range of post-embryonic developmental stages of *G. rostochiensis*. This analysis demonstrated large changes in protein pattern during the transformation of preparasitic J2 into sedentary J3 and during the metamorphosis of males into adults. The changes in abundance of three muscle proteins showed that during these two events the body-wall musculature is respectively broken down and restored. It is concluded that 4th-stage male juveniles complete their metamorphosis to adults using protein resources that they have aqcuired while feeding as 2nd- and 3rd-stage juveniles. From the occurrence of several characteristic dominant proteins in the male juvenile stages it is postulated that cyst-nematodes may use a system of storage proteins to complete the different phases of their life-cycle.

Because of the large differences between the 2D-GE patterns of the developmental stages examined in Chapters 2 and 4, it was not possible to identify putative oesophageal gland proteins in these gels. Therefore, in Chapter 5 adult males and J2 are dissected into anterior parts (containing the oesophageal glands) and posterior parts (without the glands) and the protein composition of these body fragments is analysed using mini 2D-GE. This identified a single polypeptide of 34.4 kDa which was characteristic of the anterior part of both J2 and males. Although this protein may originate from an oesophageal gland, this could not be confirmed with certainty, and other explanations for its origin remain as yet equally plausible.

The second part of the investigations (Chapters 6 and 7) describes the production of monoclonal antibodies (MAbs) against antigens from *G. rostochiensis* with the aim to identify oesophageal gland proteins. In Chapter 6 mice were immunized with antigen samples derived from preparasitic J2 and fourth-stage females, and MAbs produced by hybridoma cell lines were screened with immunofluorescence microscopy for reactivity with structures within J2. This identified MAbs with various binding specificities, including 5 MAbs which reacted strongly with the subventral oesophageal glands. Unfortunately, these latter MAbs did not identify oesophageal gland proteins on Western blots of J2, indicating that their epitopes were sensitive to denaturation by the electrophoresis detergent SDS.

In Chapter 7 mice were therefore immunized with partially purified SDS-denatured proteins from J2, which had been collected by preparative continuous flow electrophoresis. This produced 12 new MAbs against the subventral glands, of which 11 reacted with proteins on Western blots of J2. Together these MAbs identified 4 proteins of respectively 30, 31, 39 and 49 kDa. Immunoelectron microscopy with one of these MAbs showed a specific binding to the contents of the secretory granules within the subventral glands of J2, indicating that these proteins are indeed the secretory products of these gland cells. It is discussed in Chapter 7 that the antibodies to the subventral gland proteins may be useful for engineering resistance to cyst-nematodes into potato by means of 'plantibody' technology.

The MAbs to the oesophageal gland proteins from Chapter 7 will be valuable tools in future studies relating to the nature of these antigens and their possible function in the host-parasite interaction. In a following thesis by colleague G. Smant results of such further studies will be presented, and this will include the identification of the abovementioned subventral gland proteins in 2D-gels of *G. rostochiensis*.

Samenvatting

Dit proefschrift presenteert de resultaten uit een onderzoek waarin gezocht is naar de speekseleiwitten van het aardappelcysteaaltje *Globodera rostochiensis*. Dit onderzoek is verricht bij de Vakgroep Nematologie van de Landbouwuniversiteit te Wageningen.

Aardappelcysteaaltjes (*G. rostochiensis* en *G. pallida*) vormen een belangrijke plaag voor de aardappelteelt in Nederland (en ook daarbuiten) omdat zij aardappelmoeheid veroorzaken. Deze microscopisch kleine nematoden parasiteren op de wortels van de aardappelplant en kunnen hierdoor aanzienlijke opbrengstverliezen toebrengen aan dit gewas. Aardappelcysteaaltjes ontlenen hun naam aan de zogenaamde cysten (roodbruine kraaltjes van 0.5 mm) die zij vormen op de wortels van hun waardplant. Deze cysten blijven na de teelt van het gewas achter in de grond, en bevatten honderden eieren met jonge aaltjes in rusttoestand. In deze cysten kunnen de aaltjes desnoods enkele jaren overleven zonder waardplant. Zodra er echter aardappels worden geteeld kruipen de aaltjes (als 2e-stadium juvenielen) uit de cysten en dringen zij de jonge wortels van hun waardplant binnen. Hier nestelen zij zich vast, en vormen zij bestaande wortelcellen om tot een gespecialiseerd voedingscelcomplex (het syncytium) waarmee zij voedingssappen onttrekken aan hun waardplant. Tijdens hun groei in de wortel maken de aaltjes nog twee ontwikkelingsstadia door (3e en 4e juveniele stadium) voordat zij volwassen worden. De volwassen wijfjes zwellen enorm op en breken uiteindelijk als witte bolletjes door aan het worteloppervlak. Geleidelijk aan sterven de wijfjes dan af en veranderen zij in bruine cysten, waarmee de levenscyclus is voltooid.

De vorming van een voedingscelcomplex is een onmisbaar onderdeel van de levenscyclus van cysteaaltjes: zonder dit syncytium kunnen zij niet groeien en sterven zij af zonder volwassen te worden. Het sterke vermoeden bestaat dat cysteaaltjes hun speekseleiwitten gebruiken om deze voedingscellen in de wortels van hun waardplant aan te maken. Ook tijdens de groei van de aaltjes spelen speekseleiwitten waarschijnlijk een belangrijke rol bij de voedselopname uit de voedingscel. De identificatie van deze speekseleiwitten is vanuit wetenschappelijk oogpunt hoogst interessant, omdat dit inzicht geeft over de processen die plaatsvinden bij de vorming en benutting van voedingscellen door cysteaaltjes. Daarnaast kan de kennis over deze eiwitten belangrijk zijn voor het verkrijgen van nieuwe vormen van resistentie tegen aardappelmoeheid bij de aardappel.

In het eerste deel van het onderzoek (hoofstukken 2 t/m 5) zijn de eiwitten van het aardappelcysteaaltje bestudeerd met behulp van twee-dimensionale gel elektroforese (2D-GE). Met deze analysemethode worden de eiwitten afkomstig uit fijngemalen aaltjes van elkaar gescheiden door ze met behulp van elektrische spanning een gel in te laten bewegen. Eerst worden de eiwitten in één richting van elkaar gescheiden op grond van hun elektrische lading (ofwel iso-elektrisch punt), en vervolgens in de richting die hier loodrecht op staat nogmaals gescheiden op grond van hun grootte (ofwel molekuulgewicht). Na deze dubbele scheiding wordt de gel gekleurd met een zilverhoudende oplossing waardoor een patroon van verscheidene honderden eiwitvlekjes zichtbaar wordt.

In hoofdstuk 2 zijn van twee niet-parasitaire stadia (2e stadium juvenielen en mannetjes) en twee parasitaire stadia (4e stadium wijfjes en volwassen wijfjes) van *G. rostochiensis* de eiwitpatronen vergeleken. Er waren grote verschillen in eiwitsamenstelling tussen deze vier ontwikkelingsstadia, waarbij relatief de meeste overeenkomsten werden gevonden tussen eiwitpatronen van beide niet-parasitaire stadia (67.4%) en beide parasitaire stadia (76.7%).

In hoofdstuk 3 wordt een gevoelige mini 2D-GE techniek gepresenteerd, waarmee het mogelijk is om elektroforese patronen te verkrijgen van zeer kleine hoeveelheden eiwit. De gevoeligheid van deze elektroforese techniek wordt gedemonstreerd aan de hand van een genetische analyse van individuele wijfjes van *G. pallida*.

In hoofdstuk 4 wordt deze mini 2D-GE techniek toegepast om een eiwitanalyse van de allerkleinste parasitaire ontwikkelingsstadia van *G. rostochiensis* uit te kunnen voeren. Van opeenvolgende ontwikkelingsstadia, te weten eieren, 2e, 3e, en 4e stadium juvenielen en de volwassen aaltjes, zijn de eiwitpatronen vergeleken. Hieruit bleek dat de grootste veranderingen in eiwitsamenstelling optreden bij het parasitair worden van de aaltjes en tijdens de laatste ontwikkelingsfase (de 'metamorfose') van de mannelijke cysteaaltjes. Aan de hand van de aanwezigheid van enkele spiereiwitten is aangetoond dat de aaltjes na het binnendringen in de wortel hun spiermassa afbreken. Ook is aangetoond dat volwassen mannetjes vóór het verlaten van de wortel hun spiermassa weer opbouwen, en dat zij hiervoor waarschijnlijk de eiwitvoorraad aanspreken die zij tijdens hun voorafgaande voedingsperiode hebben opgebouwd. De metamorfose van de mannelijke cysteaaltjes vertoont hierin veel gelijkenis met de verpopping van insekten.

Vanwege de grote verschillen in eiwitsamenstelling tussen de ontwikkelingsstadia van *G. rostochiensis* kon in de eiwitpatronen van hoofdstuk 2 en 4 geen zinvolle selektie van mogelijke speekseleiwitten worden gemaakt. Daarom is in hoofdstuk 5 begonnen met de eiwitanalyse van doorgesneden aaltjes: 2e stadium

juvenielen en volwassen mannetjes van *G. rostochiensis* zijn gescheiden in voorkanten (die de speekselklieren bevatten) en achterkanten (zonder speekselklieren) en de eiwitten in deze lichaamsdelen zijn na mini 2D-elektroforese met elkaar vergeleken. Er kon één eiwit met een molekuulgewicht van 34.4 kDa worden aangewezen dat in beide ontwikkelingsstadia specifiek in de voorkant voorkwam. Hoewel dit mogelijk een eiwit uit een speekselklier is, kon dit niet met zekerheid worden vastgesteld, en andere verklaringen voor de herkomst van dit eiwit blijven vooralsnog even waarschijnlijk.

In het tweede deel van het onderzoek (hoofdstukken 6 en 7) zijn monoklonale antilichamen gemaakt tegen 2e-stadium juvenielen (J2's) van het aardappelcysteaaltje, met de bedoeling om via een andere weg tot een identificatie van speekseleiwitten te komen. Antilichamen (ook wel antistoffen genoemd) zijn eiwitten die worden gevormd door het afweersysteem van (onder andere) zoogdieren om ziekteverwekkers en lichaamsvreemde stoffen te bestrijden. Deze antilichamen worden 'op maat' door witte bloedcellen aangemaakt en binden zeer specifiek aan de ziekteverwekker of lichaamsvreemde stof waartegen zij gericht zijn. Voor het verkrijgen van de antilichamen in hoofdstuk 6 en 7 zijn muizen ingespoten ('geimmuniseerd') met eiwitten van het aardappelcysteaaltje. Zodra de muizen voldoende antilichamen tegen deze eiwitten hadden gevormd, werden witte bloedcellen uit de milt verzameld en in het laboratorium als zogenaamde 'hybridoma' cellijnen in kweek gebracht. Elk van deze hybridoma cellijnen produceert één zogenaamd 'monoklonaal' antilichaam dat gericht is tegen één enkel eiwit van het aardappelcysteaaltje. De monoklonale antilichamen worden door de hybridoma cellen afgegeven aan het voedingsmedium waarin zij groeien. Door nu dit voedingsmedium aan opengesneden aaltjes toe te voegen en vervolgens te 'kleuren' met een fluorescerend (= lichtgevend) tweede antilichaam dat specifiek bindt aan alle muize-antilichamen, kan met behulp van een fluorescentie microscoop gekeken worden of het antilichaam van de desbetreffende hybridoma bindt aan de speekselklieren. Op deze manier zijn in totaal enkele duizenden 'monoklonalen' getest op hun reaktie met het J2's van aardappelcysteaaltje.

In hoofdstuk 6 zijn, na immunisaties met diverse eiwitpreparaten van J2's, monoklonale antilichamen verkregen die reageren met uiteenlopende structuren van het aaltje zoals de subventrale speekselklieren, de lichaamswandspieren, het cuticula oppervlak (= de buitenkant), het darmkanaal, en de celkernen. Er is gekeken of met deze antilichamen het bijbehorende eiwit in gel elektroforese patronen kon worden geïdentificeerd. Met de antilichamen tegen de spieren konden zo inderdaad eiwitten worden geïdentificeerd. De antilichamen tegen de speekselklieren wilden echter niet binden aan eiwitten die met elektroforese waren gescheiden. Het vermoeden bestond dat de speekseleiwitten door de

elektroforese dusdanig waren gedenatureerd (= beschadigd) dat zij niet meer door de antilichamen werden herkend. Daarom werd in hoofdstuk 7 een nieuwe strategie toegepast: muizen werden nu ingespoten met aaltjeseiwitten die met behulp van elektroforese waren voorgezuiverd, en die dus reeds vooraf waren gedenatureerd. Dit leverde 12 nieuwe monoklonale antilichamen op gericht tegen de subventrale speekselklieren, waarvan er nu maar liefst 11 reageerden met elektroforese patronen van J2's. Met deze antilichamen konden in totaal 4 eiwitten worden geïdentificeerd, met molekuulgewichten van respectievelijk 30, 31, 39 en 49 kDa. Eén van deze antlichamen is gebruikt om, met behulp van immuno-goud labelling, in elektronenmicroscopische preparaten van J2's de precieze locatie van deze eiwitten in de subventrale speekselklieren vast te stellen. Het bleek dat dit antilichaam reageerde met de inhoud van de secretieblaasjes in deze klieren. Dit betekent dat deze eiwitten waarschijnlijk inderdaad speekseleiwitten uit de subventrale klier van het aardappelcysteaaltje zijn. In hoofdstuk 7 wordt toegelicht dat de antilichamen tegen de deze eiwitten wellicht kunnen worden ingezet voor het verkrijgen van resistentie tegen aardappelcysteaaltjes door middel van 'plantibody' technologie.

Met het beschikbaar komen van monoklonale antilichamen die reageren met eiwitten van de subventrale speekselklieren van het aardappelcysteaaltje is een doorbraak bewerkstelligd in het molekulair onderzoek aan plant-parasitaire aaltjes. De weg ligt nu open voor verder onderzoek naar de aard en de funktie van deze speekselkliereiwitten en naar hun genetische code. In een volgend proefschrift van collega G. Smant zullen resultaten van dergelijk verder onderzoek (waaronder de identificatie van bovengenoemde speekselkliereiwitten in twee-dimensionale eiwitpatronen van *G. rostochiensis*) worden gepresenteerd.

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Curriculum Vitae

Jan Marinus de Boer werd 7 mei 1958 geboren in Hoorn (Noord-Holland). Van 1970 tot en met 1976 volgde hij de opleiding Atheneum-B aan de Rijksscholengemeenschap "West-Friesland" te Hoorn. In 1976 werd begonnen met de studie Biologie aan de Universiteit van Amsterdam. Na het kandidaatsdiploma (april 1980) werden tijdens de doctoraalstudie de vakken Experimentele Entomologie, Aquatische Oecologie en Fytopathologie gevolgd, waarna het doctoraal diploma (cum laude) werd behaald in februari 1984. Na het volgen van een cursus elecronenmicroscopie was hij van mei 1984 tot juli 1986 werkzaam bij de Vakgroep Electronenmicroscopie en Moleculaire Cytologie van de Universiteit van Amsterdam, alwaar - in samenwerking met een vrouwenarts - de aanhechting van beweeglijke komma-vormige bacteriën (*Mobiluncus* spp.) aan vaginale epitheelcellen werd bestudeerd. In augustus 1986 werd hij als wetenschappelijk assistent aangesteld bij de Vakgroep Nematologie van de Landbouwuniversiteit. Per september 1989 werd deze aanstelling gecontinueerd door financiering van de Nederlandse Aardappel Associatie. Sinds juli 1991 is hij als projectmedewerker in dienst bij de Nederlandse Organisatie voor Wetenschappelijk Onderzoek en gestationeerd bij de Vakgroep Nematologie.

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