Species-Specific and Thermostable Proteins from Second-Stage Larvae of *Globodera rostochiensis* and *G. pallida*

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ABSTRACT

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Different electrophoretic techniques were applied to extracts of second-stage larvae of six *Globodera rostochiensis* and six *G. pallida* populations. Electrophoresis of native proteins clearly distinguished *G. rostochiensis* from *G. pallida*. Four major protein bands were specific for *G. rostochiensis* and five for *G. pallida*. However, repeated experiments gave large variations in intensities of most of the species-specific protein bands. The species-specific protein bands resolved with sodium dodecyl sulfate (SDS) electrophoresis were more reproducible. In contrast with other reports, no consistent intraspecific variation could be detected with one-dimensional electrophoresis. Several of the species-specific proteins resolved with SDS electrophoresis appeared to be thermostable and were partially purified. Characterization of the thermostable polypeptides by two-dimensional electrophoresis (20) resolved three polypeptides specific for *G. rostochiensis*, with isoelectric points (pI) and molecular masses of 20.6 kDa (pI 5.30), 20.8 kDa (pI 5.20), and 18.0 kDa (pI 6.00); these differed slightly from those specific for *G. pallida*, with pI and molecular masses of 21.0 kDa (pI 5.32), 20.5 kDa (pI 5.40), and 17.0 kDa (pI 5.80).

Additional key words: pathotypes, potato cyst nematodes, silver stain.

Together with crop rotation and the use of nematicides, resistant potato cultivars are essential for the control of the potato cyst nematodes *Globodera rostochiensis* (Woll.) Behrens and *G. pallida* (Stone) Behrens. Resistance is mainly derived from *Solanum tuberosum* ssp. *andigena* Juz. and Buk. (28) and *S. vernei* Bitt. and Wittm. (10), and is mediated by pathotype-specific genes (3,15,23,31). However, the commercial success of resistant cultivars is limited by the widespread occurrence of virulent cyst nematode populations. Eight pathotypes of the potato cyst nematodes (15) are recognized, five within *G. rostochiensis* (Ro_1–Ro_5) and three within *G. pallida* (Pa_1–Pa_3). Because large areas are infested with both species, current breeding programs are mainly focused on resistance against most or all pathotypes of both species, which is a major constraint for the introduction of new, resistant cultivars.

A reliable and quick screening test able to characterize field infestations of potato cyst nematodes according to species should improve possibilities for control by means of resistance. Diagnosis of field populations will allow growers to select cultivars with resistance against either of the two species, and the introduction of new, resistant cultivars will be facilitated by incorporating genes for resistance effective against either *G. rostochiensis* or *G. pallida*. The low dispersal abilities and the relative low reproduction rate of the potato cyst nematodes favor such an approach. Moreover, selection towards alleles for virulence is rather slow (11–13,31), and in many fields, resistant cultivars can be grown without loss of yield for several years.

Identification of *G. rostochiensis* and *G. pallida* by morphological measurements is hampered by intraspecific variation and interspecific overlap (25–27), and several morphological characteristics are influenced by such environmental factors as temperature and availability of food (4). Morphological differentiation of these sibling species is therefore time-consuming and not suited for large-scale routine purposes.

The objective of this study was to trace species-specific proteins that have suitable properties for the development of a serological assay. In addition, we speculate on the possibility for pathotyping field populations.

MATERIALS AND METHODS

Populations. Twelve populations of *G. rostochiensis* and *G. pallida* were obtained from different geographical sources (Table 1). Populations 1–5, 7, 9, and 10 were supplied by Ir. C. Miller and Ing. J. Bakker, Plant Protection Service, Wageningen, the Netherlands; population no. 12 by Ir. A. Mulder, Hilbrands Laboratorium, Assen, the Netherlands; populations 6 and 11 by Dr. H. J. Rumpenhorst, Department of Nematology, Muenster, Federal Republic of Germany; and population no. 8 by Dr. A. R. Stone, Rothamsted Experimental Station, Harpenden, England. All populations were maintained on *S. t.* ssp. *tuberosum* 'Eigenheimer,' susceptible to all pathotypes.

Morphological characterization. Second-stage larvae were killed in FP-4 (18) and fixed in 4% cold formalin. Further processing for measurements was as described by Seinhorst (22). On the average, 20 specimens per population were measured by our taxonomist, Dr. P. A. A. Loof.

Virulence characterization. The populations were tested for their reproductive ability on the differentials used in the international pathotype scheme of Kort et al (15). *S. t.* ssp. *tuberosum* was represented by commercial cultivars susceptible to all pathotypes. *S. t.* ssp. *andigena* was replaced by the commercial cultivar Saturna, which has the resistance gene H1 derived from CPC 1673. In experiments in petri dishes done by our geneticist, Ir. R. Janssen, *S. vernei* hybrid 62.33.3 was replaced by cultivar Darwina. *S. kurtzianum* hybrid 60.21.19 and *S. multidissectum* hybrid P55/7 were not included in this test.

Virulence characteristics were measured by inoculating 50

second-stage larvae (two per root tip) on roots of sprouts grown in petri dishes with water-agar (17). The number of females that appeared on the roots of the differential was expressed as percentage of the number of females on the potato cultivar Eigenheimer. This percentage, the relative number of females, was used as an indication for the number of virulent genotypes in a population.

In several cases, the virulence characteristics in populations were estimated from the P_f/P_i values on the differentials by the Plant Protection Service's Miller and Bakker, and expressed as a percentage of the P_f/P_i on the potato cultivar Mentor or a comparable genotype susceptible to all pathotypes. For these tests, potato plants were grown in pots (8-cm diameter, 150 ml volume) filled with sandy loam, and inoculated with 25 cysts (P_i). Final cyst counts (P_f) were determined after a growing period of 2 mo.

Preparation of samples. Second-stage larvae were hatched from approximately 300 cysts with potato root diffusate. The larvae were collected every 2 days and stored at 4 C. Four days after hatching, viable larvae were allowed to move through a cotton wool filter, and approximately 40,000 individuals were concentrated by centrifuging (1,000 *g*), rinsed three times with 20 ml of 10 mM Tris-HCl, pH 7.4, and suspended in 200 μl of 10 mM Tris-HCl, pH 7.4.

Nematodes were homogenized on ice in a 2-ml glass Potter homogenizer with a tightly fitting Teflon pestle (clearance capacity 25 μm) at 1,700 rpm, 15 times for 20 sec, with 10-sec intervals. This treatment completely disrupted the nematodes, as confirmed with a dissecting microscope.

Polyacrylamide gel electrophoresis of native proteins. Nematodes were homogenized in 10 mM Tris-HCl, pH 7.4. The homogenate was centrifuged for 10 min at 105,000 *g*. Protein determinations in the supernatant were made according to Bradford (2) by measuring the shift in extinction of Coomassie Brilliant Blue at 595 nm. The supernatant was treated with 10% glycerol (w/v) and 0.01% bromphenol blue and immediately used for electrophoresis.

Native polyacrylamide gel electrophoresis was done at 4 C in a slab gel apparatus (model 220, Bio-Rad, Richmond, CA) with 3-mm slots, a 3% acrylamide stacking gel, and a 7% acrylamide separation gel (1.5-mm thickness). The current was 10 mA for 30 min and continued at 20 mA. The buffer system was essentially that according to Laemmli (16), but sodium dodecyl sulfate (SDS) was omitted. Protein band positions were expressed as Rf values calculated by dividing the migration distance of the protein by the migration distance of bromphenol blue. The bromphenol blue marker was given an Rf value of 1.00.

SDS polyacrylamide gel electrophoresis. Soluble protein extracts were prepared by homogenizing the nematodes in 10 mM Tris-HCl, pH 7.4, 5% 2-mercaptoethanol (v/v). The homogenate was centrifuged at 105,000 *g*. Sixty μl of supernatant was saturated with 64 mg of urea and stored at −80 C until use. Thermostable proteins were isolated as follows. Second-stage larvae were homogenized in 10 mM Tris-HCl, pH 7.4, and the crude homogenate was heated at 100 C for 10 min. Most of the proteins (approximately 98%) were precipitated by centrifuging (105,000 *g*). The thermostable proteins remained in solution. Sixty μl of supernatant was saturated with 64 mg of urea and stored at −80 C until use. Protein samples saturated with urea could be stored at −80 C for at least two years without detectable changes.

Before electrophoresis, 13 μl of 10 mM Tris-HCl, pH 7.4, 5% 2-mercaptoethanol (v/v) was added to the thawed sample. After centrifugation (105,000 *g*) and determination of protein concentrations, an equal volume of buffer of the following composition was added: 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2.3% (w/v) SDS, and 0.01% (w/v) bromphenol blue in 62.5 mM Tris-HCl, pH 6.8.

SDS polyacrylamide slab gels were prepared according to Laemmli (16) with a 6% acrylamide stacking gel and a 12% acrylamide separation gel (1.5-mm thickness). Electrophoresis was done at 20 mA for 30 min and continued at 35 mA.

Apparent molecular weights were estimated by using phosphorylase B, bovine serum albumine, ovalbumine, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme as reference proteins (Bio-Rad low-molecular weight standard solution).

Two-dimensional gel electrophoresis. Nematodes were homogenized in 10 mM Tris-HCl, pH 7.4, 5% (v/v) 2-mercaptoethanol. The homogenate (60 μl) was saturated with 64 mg of urea and stored at −80 C. The isolation of thermostable proteins was similar to that described above.

Before electrophoresis, 13μl of a mixture of 5 μl ampholines, pH 3–10, 20μl ampholines, pH 4–6 (Biolyte, Bio-Rad), 50 μl 20% Nonidet P-40 (Sigma Chemical Co., St. Louis, MO), and 25 μl 2-mercaptoethanol was added to the thawed sample. The sample was centrifuged (105,000 *g*) and used immediately for isoelectric focusing.

Isoelectric focusing in the pH region of 4–6 was done essentially as described by O'Farrell (20), with the following modifications. The ampholines, pH 5–7, were replaced by ampholines, pH 4–6, and gels were polymerized in glass tubes (160 mm × 2 mm inside diameter). The sample (25–50 μl) was loaded without prefocusing. Isoelectric focusing was done according to the following schedule: 30 min, 100 V; 30 min, 200 V; 15 hr, 300 V; and 3 hr, 400 V. In the second dimension, the proteins were separated in SDS polyacrylamide gels as described above but with a constant current of 25 mA. Between isoelectric focusing and SDS electophoresis, gels were equilibrated for 10 min in 10% (w/v)

TABLE 1. Geographical source and virulence characteristics in the *Globodera rostochiensis* (Ro) and *G. pallida* (Pa) populations

Population no.	Pathotype, collection location, and code[a]	Relative number of females[b]					
		S. tuberosum spp. *andigena* hybr. CPC 1673		*S. vernei* hybr. 58.1642/4		*S. vernei* hybr. 62.33.3	
1	Ro_1, Wageningen, the Netherlands, MIER	0	(−)	1 ± 0.2[c]	(−)	0	(−)
2	Ro_1, Weert, the Netherlands, A-13	0	(−)	1 ± 1.3[c]	(−)	0	(−)
3	Ro_1, Hardenberg, the Netherlands, C-133	1.4 ± 0.1	(−)	0.3 ± 0.2[c]	(−)	0	(−)
4	Ro_3, Hoogeveen, the Netherlands, C-129	30.0 ± 1.5	(+)	2.2 ± 2[c]	(−)	0.5 ± 0.4[c]	(−)
5	Ro_4, Emmen, the Netherlands, F-515	0	(−)	10 ± 5[c]	(+)	0	(−)
6	Ro_5, Harmerz, Federal Republic of Germany, H	84.6 ± 3.7	(+)	30 ± 9[c]	(+)	15.9 ± 1.7	(+)
7	Pa_1, Glarryford, Northern Ireland, 1337	...[d]	(+)	...[d]	(+)	10.4 ± 2.5[c]	(−)
8	Pa_2, New Leake, Great Britain, ST	...[d]	(+)	...[d]	(+)	3.3 ± 2[c]	(−)
9	Pa_3, Cadishead, Great Britain, E-1202	...[d]	(+)	...[d]	(+)	6.7 ± 0.7	(+)
10	Pa_3, Far Oer, Denmark, E-1215	...[d]	(+)	...[d]	(+)	9.3 ± 0.7	(+)
11	Pa_3, Frenswegen, Federal Republic of Germany, FR	...[d]	(+)	...[d]	(+)	5.7 ± 0.6	(+)
12	Pa_2, Veendam, the Netherlands, HPL-1	...[d]	(+)	...[d]	(+)	1.5 ± 0.5	(−)

[a] As designated in the original collections.
[b] Numbers of females produced on differentials expressed as percentages of those on susceptible plants ± the standard deviations; (−) and (+) refer to combinations that are classified according to the international pathotype scheme as incompatible and compatible, respectively.
[c] Data obtained from pot experiments.
[d] Estimates not made.

glycerol, 5% (v/v) 2-mercaptoethanol, and 2.3% (w/v) SDS in 62.5 mM Tris-HCl, pH 6.8. The isoelectric points (pI) of the polypeptides were estimated by measuring the pH profile of the isoelectric focusing gel with a surface pH electrode (Bio-Rad). The standard deviations of these pI values averaged 0.1 pH per unit.

Staining. Staining with silver was done according to Oakley et al (19). In several experiments, proteins were stained with Coomassie Brilliant Blue R250. The staining solution contained 0.2% Coomassie Brilliant Blue, 50% (v/v) methanol, and 7% (v/v) acetic acid. The gels were destained in 5% (v/v) methanol and 7% (v/v) acetic acid.

RESULTS

To define our populations as well as possible, we examined the variation in virulence and morphology. In most combinations designated as compatible according to the international pathotype scheme (15), the number of females that developed on the differentials was remarkably low when compared with the general susceptible host (Table 1). For example, the relative number of females produced on *S. vernei* hybrid 62.33.3 by the Pa_3 populations 9, 10, and 11 did not exceed 10%. This indicates that these populations are mixtures of individuals differing in their ability to overcome resistance. Furthermore, populations classified as virulent for the same differential differ in their ability to overcome resistance. For example, populations 4 and 6, both classified as virulent for *S. t.* ssp. *andigena*, have a value of 30.0% and 84.6%, respectively, for the relative number of females produced on *S. t.* ssp. *andigena*.

Morphological characteristics of second-stage larvae are given in Table 2. Body length and tail length differ markedly from the values given in the original descriptions of *G. rostochiensis* and *G. pallida* (25) and are not informative in discriminating the two species. Stylet length and distance from median bulb valve to excretory pore are in concordance with the original descriptions. Although these values are variable and overlap between the two species, the average values clearly discriminate the two species. The species designation of the populations is also supported by the shape of the stylet knobs (Table 1) and the color of the females (data not shown).

The 12 populations were investigated with different types of protein electrophoresis. Electrophoresis of native proteins clearly distinguishes the two species (Fig. 1). Protein bands with Rf values of 0.350, 0.595, 0.650, and 0.750 are specific for *G. rostochiensis* and Rf values of 0.240, 0.550, 0.590, 0.760, and 0.780 are specific for *G. pallida*. The intensities of several species-specific protein bands (e.g., with Rf values of 0.350, 0.650, and 0.760) varied between repeated experiments, probably due to a combination of small variations in the extraction procedures (e.g., time between homogenization and electrophoresis) and the instability of the native proteins. In some cases, species-specific protein bands were even fully absent (e.g., with Rf values of 0.350, populations 3 and 6) while present in other experiments with the same population. No consistent intraspecific differences were detectable. Storage of native protein samples at −80 C caused many of the proteins to detoriate, resulting in diffuse bands after electrophoresis (results not shown).

Figure 2 compares the SDS denaturated protein profiles of four *G. rostochiensis* populations and of four *G. pallida*. Three major protein bands with molecular masses of 31.0 kDa, 20.7 kDa, and 18.0 kDa were specific for *G. rostochiensis*, and four major bands with molecular masses of 31.5 kDa, 21.0 kDa, 20.5 kDa, and 17.0 kDa were specific for *G. pallida*. Subtle quantitative differences are also seen in diffuse bands. These bands probably consist of several polypeptides differing slightly in molecular masses. The intensities of these bands varied between repeated experiments, and therefore are not suited for species identification. In Figure 2, only highly reproducible species-specific bands are indicated. The protein bands with molecular masses of 20.7 kDa, 18.0 kDa, 21.0 kDa, 20.5 kDa, and 17.0 kDa stain grey with silver, whereas most of the proteins stain brown or reddish brown. Protein profiles visualized with Coomassie Brilliant Blue (Fig. 3) again demonstrate that some of the species-specific proteins are major components of the total soluble protein fraction. No intraspecific differences were found with one-dimensional SDS electrophoresis, neither qualitative nor quantitative.

Figure 3 shows the SDS protein patterns of total soluble protein fractions and thermostable proteins from second-stage larvae. The heat treatment resulted in an approximately 50-fold purification of some species-specific proteins (with molecular masses of 20.7 kDa, 18.0 kDa, 21.0 kDa, 20.5 kDa, and 17.0 kDa). The species-specific thermostable proteins have a high affinity for the silver stain. Application of 10 ng of heat-treated protein is sufficient to resolve the species-specific proteins.

The heat-stable proteins were further characterized by combining isoelectric focusing and SDS polyacrylamide gel electrophoresis. Two-dimensional electrophoretic patterns of total protein and partially purified proteins are shown in Figure 4. The species-specific thermostable proteins are marked by their molecular masses in kDa. The 20.7-kDa protein band, specific for *G. rostochiensis*, actually consists of two polypeptides with molecular masses of 20.6 kDa and 20.8 kDa (Fig. 4A and C). Two-dimensional electrophoresis of an equal mixture of heat-stable proteins of the two species shows the small differences in pI (Fig. 5). The specific polypeptides of *G. rostochiensis*, with molecular masses of 20.6 kDa and 20.8 kDa, have a pI of 5.30 and 5.20, respectively. The specific thermostable proteins of *G. pallida*, with molecular masses of 20.5 kDa and 21.0 kDa, have a slightly higher pI, 5.40 and 5.32, respectively. The polypeptide with a molecular mass of 18.0 kDa, specific for *G. rostochiensis*, has a pI of 6.00, and the 17.0 kDa polypeptide of *G. pallida* has a pI of 5.80 (Fig. 5). The differences between the pI values of the species-

TABLE 2. Morphological characteristics of second-stage larvae of *Globodera rostochiensis* (1-6) and *G. pallida* (7-12)

Population no.[a]	Stylet length (μm)[b]	Body length (μm)[b]	Median bulb valve to excretory pore (μm)[b]	Tail length (μm)[b]	Stylet[c] knobs
1	21.0 ± 0.6	453 ± 19	31.6 ± 2.0	50.0 ± 2.9	16 R, 9 R-P
2	20.9 ± 0.5	438 ± 18	32.5 ± 3.5	50.0 ± 4.4	14 R, 1 R-P
3	20.8 ± 0.6	436 ± 19	33.5 ± 2.1	48.8 ± 2.5	9 R, 5 R-P, 3 P
4	20.7 ± 0.6	420 ± 22	30.6 ± 2.8	47.5 ± 2.7	20 R, 5 R-P
5	21.4 ± 0.9	449 ± 18	32.6 ± 2.9	45.5 ± 2.4	19 R, 1 R-P
6	21.0 ± 0.5	442 ± 20	29.3 ± 3.2	48.1 ± 3.7	14 R, 1 R-P
7	22.4 ± 1.1	459 ± 30	36.3 ± 4.0	49.0 ± 4.8	17 P, 4 R-P
8	22.4 ± 0.8	448 ± 19	38.8 ± 2.4	48.9 ± 3.5	20 P
9	23.6 ± 0.9	449 ± 23	37.4 ± 2.8	50.2 ± 3.8	18 P, 2 R-P
10	22.0 ± 0.5	418 ± 17	35.2 ± 2.6	47.3 ± 2.5	20 P
11	22.8 ± 0.8	459 ± 19	37.2 ± 3.0	49.2 ± 3.7	25 P
12	23.3 ± 0.8	458 ± 18	39.0 ± 4.3	51.1 ± 2.7	19 P, 1 R-P

[a] Numbers refer to the populations in Table 1.
[b] ± standard deviations.
[c] Numbers of specimens with basal stylet knobs rounded (R) and pointed anteriorly (P), as described for *G. rostochiensis* Rostoch and *G. pallida* Epworth, respectively (25); R-P indicates an intermediate shape.

specific proteins are constant, whereas the estimates of the absolute pI values varied between independent measurements.

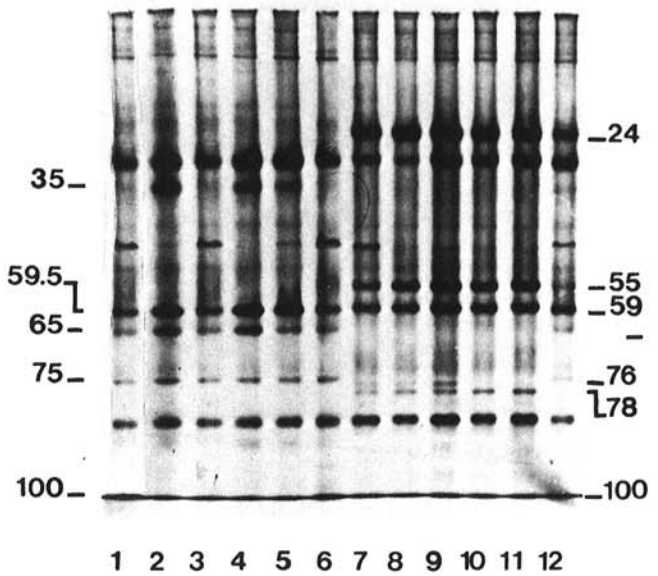

Fig. 1. Electrophoresis under native conditions of second-stage larvae of six *G. rostochiensis* populations (1–6) and six *G. pallida* populations (7–12). The species-specific protein bands are indicated with their Rf values ($\times 10^2$). The migration distance of bromphenol blue was given an Rf value of 1.00. Numbers at the bottom of the lanes refer to the populations in Table 1. Proteins were stained with silver. Samples contained 3 μg of protein.

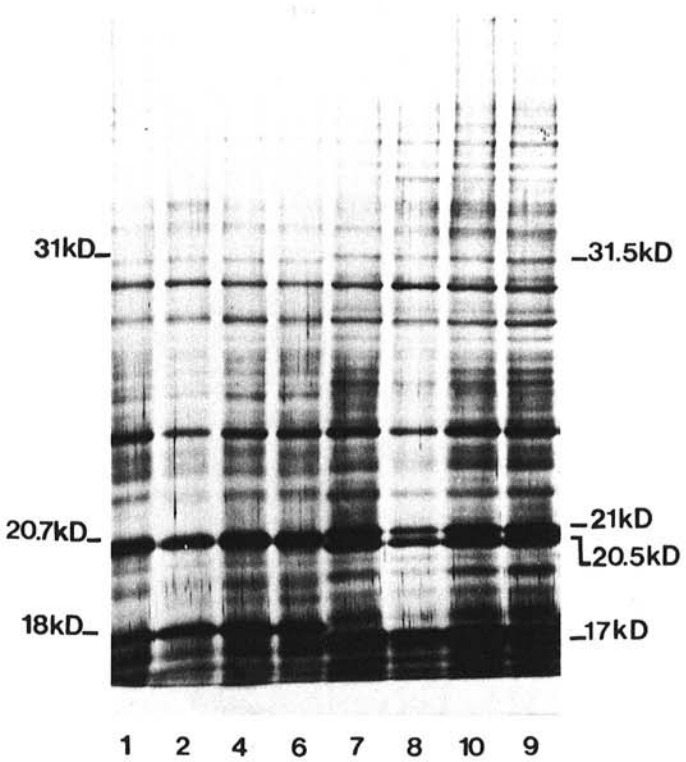

Fig. 2. SDS electrophoresis of second-stage larvae of *G. rostochiensis* (1, 2, 4, 6) and *G. pallida* (7–10). Species-specific protein bands are marked. Values at the left and right of the figure refer to molecular masses in kilodaltons. Numbers at the bottom of the lanes refer to the populations in Table 1. Proteins were stained with silver. Samples with 3 μg of protein were used.

The species-specific proteins showed no detectable changes in molecular mass or pI after the partial purification (Fig. 4), indicating that the proteins are not markedly affected by the heat treatment. The thermostable proteins exhibited no intraspecific variation as was assessed by two-dimensional electrophoresis of all populations (data not shown).

DISCUSSION

Interspecific variation. A variety of electrophoretic techniques have been used with crude homogenates of *G. rostochiensis* and *G. pallida*. Using these methods, several authors have reported distinct protein differences between the two species (1,5–9,21,24,29,30,32). So far, the most promising approach as an advisory tool has been to use isoelectric focusing of native proteins of eggs and cysts (5,6). Combined with a densitometric evaluation of the protein concentrations it was possible to quantify proportions of *G. rostochiensis* and *G. pallida* in mixed samples (5,6). However, electrophoresis is probably too laborious and too expensive to use as a routine advisory tool.

Our approach was to search for species-specific proteins having suitable properties for the development of a serological assay. Heat treatment of crude homogenates resolved several species-specific

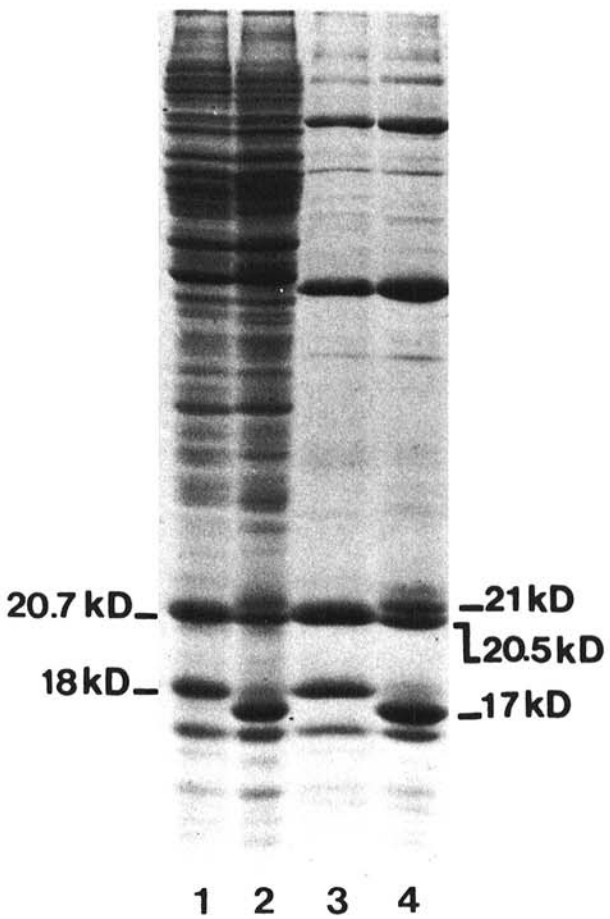

Fig. 3. SDS electrophoresis of crude homogenates (lanes 1 and 2) and thermostable proteins (lanes 3 and 4). The molecular masses of the thermostable proteins specific for *G. rostochiensis* (lanes 1 and 3) and *G. pallida* (lanes 2 and 4) are indicated in kilodaltons at the left and right of the figure, respectively. Samples of total soluble protein (30 μg) and thermostable protein (5 μg) were obtained from populations 1 and 8 (see Table 1). Proteins were stained with Coomassie Brilliant Blue.

proteins (Figs. 3 and 4C and D), which have desirable characteristics for the preparation of specific antibodies. These easily purified proteins are similar in molecular mass, pI, and color when stained with silver. The thermostable proteins seem to be excellent targets to quantify proportions of *G. rostochiensis* and *G. pallida* in mixed samples by measuring concentrations of comparable proteins in the two species. This ability to quantify proportions may be crucial for the success of a diagnostic test, because field infestations are often mixtures of both species (14). Another desirable trait is that these species-specific proteins are major components of the soluble protein fraction, which would facilitate the isolation of sufficient antigen. Also, none of the species-specific thermostable proteins manifested intraspecific variation, as was ascertained with two-dimensional electrophoresis of six *G. rostochiensis* and six *G. pallida* populations. Considering the diverse provenances of the populations tested (Table 1), it seems feasible to assume that the species-specific thermostable polypeptides shown in Figure 5 are representative of many *G. rostochiensis* and *G. pallida* populations in western Europe.

Intraspecific variation. Pathotypes of *G. rostochiensis* and *G. pallida* have been the focus of several electrophoretic studies (1,7–9,24). Populations, representing several pathotypes of *G. rostochiensis* and *G. pallida*, have been differentiated by conventional disc-electrophoresis of second-stage larvae (9) and by two-dimensional electrophoresis of eggs (24), revealing 10–25 major proteins. Unlike those investigators, we were unable to detect consistent intraspecific variations with one-dimensional electrophoresis (Figs. 1 and 2).

The search for pathotype-specific proteins is far more complex than implicitly suggested in the various biochemical reports dealing with pathotypes of potato cyst nematodes (1,7,9,24,32). One of the complicating factors is the way populations are classified in current pathotype schemes (3,15). Populations are designated as avirulent or virulent for a certain differential, if the reproduction factor (P_f/P_i) is ≤ 1 or > 1, respectively (3,15). Taking a reproduction factor of 20 on the general susceptible host, which is a normal value, a reproduction factor of 1 on a differential infers that approximately 5% of the individuals are capable of development on that differential (Table 1). Hence, according to this definition, the number of virulent individuals for a given differential in populations classified as identical pathotypes may vary from approximately 5% to 100%. As a consequence, current

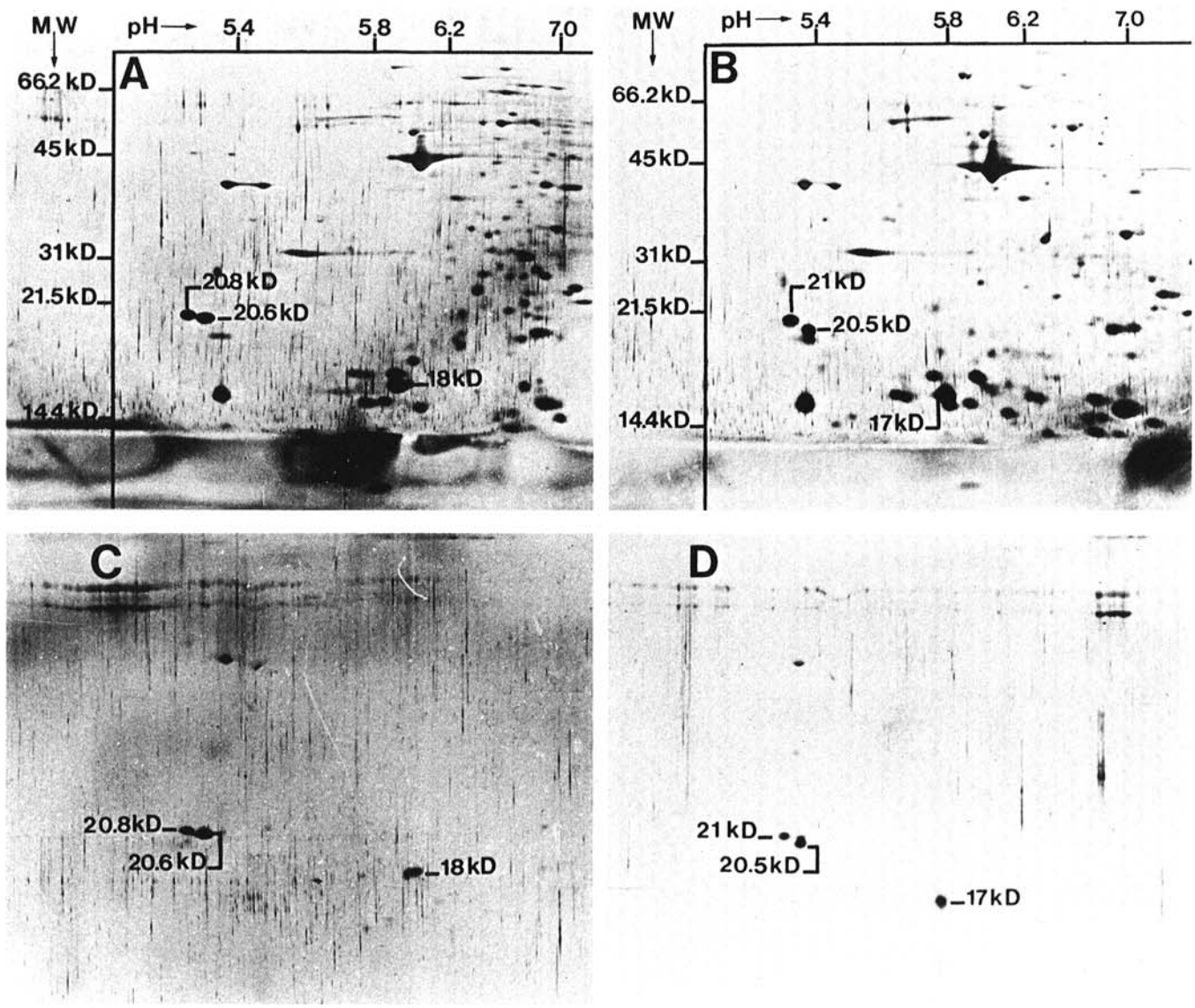

Fig. 4. Two-dimensional electrophoretic patterns of total protein (25 μg) of *G. rostochiensis* (A) and of *G. pallida* (B) and thermostable proteins (0.5 μg) of *G. rostochiensis* (C) and of *G. pallida* (D). The molecular masses of the species-specific thermostable polypeptides are given in kilodaltons. The protein band of 20.7 kDa, specific for *G. rostochiensis*, actually consists of two polypeptides, a peptide of 20.8 kDa and of 20.6 kDa. *G. rostochiensis* and *G. pallida* are represented by populations 1 and 8, respectively (see Table 1). Proteins were stained with silver.

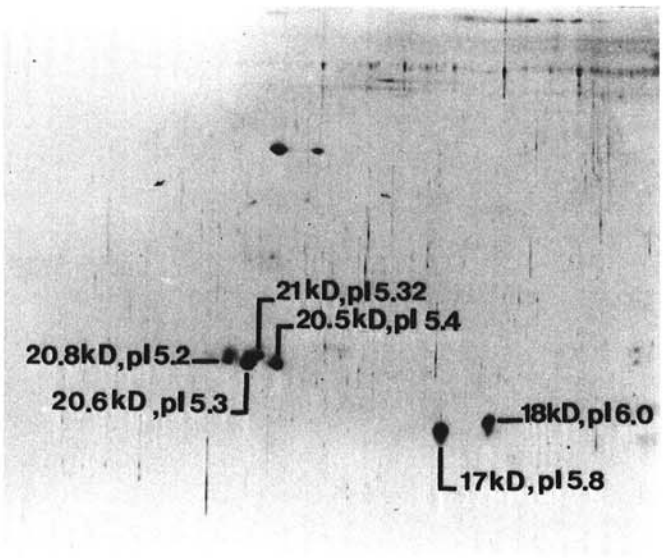

Fig. 5. Two-dimensional electrophoretic pattern of a mixture of equal protein quantities of the thermostable proteins of *G. rostochiensis* (0.25 μg) and *G. pallida* (0.25 μg). The species-specific polypeptides are indicated by their approximate isoelectric points and molecular masses in kilodaltons. Samples were prepared from populations 1 and 8 (see Table 1). Proteins were stained with silver.

pathotype classification is not accurate enough to trace correlations between intraspecific protein variations and virulent genotypes. For a proper analysis, it is necessary to estimate for each differential the number of virulent genotypes (Table 1). Until now, such estimates have never been presented in biochemical studies on pathotypes (1,7,9,24,32). In view of these considerations, existing biochemical information on intraspecific variations (1,7,9,24,32) is evidently of little use in developing tests to quantify the number of virulent genotypes in field populations.

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