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The Composition of the Cyst Wall of the Beet Cyst-Nematode *Heterodera schachtii*

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1. Cyst walls of the beet cyst-nematode (*Heterodera schachtii* Schmidt) were obtained by sieving a suspension of crushed cysts; about 15mg of dried cyst walls was obtained from 1000 cysts. 2. The cyst walls contained 68% protein calculated from nitrogen content. Glutamic acid, glycine, proline and hydroxyproline made up about 54% by weight of the amino acids obtained on acid hydrolysis. 3. Minor constituents of the cyst wall were hexosamine (3.3%), lipid (6%), carbohydrate (2%) and phenols (2%). The hexosamine was identified as galactosamine. 4. The cyst walls contained inorganic material (ash 17%), most of which was extractable with EDTA, but not with water. Major inorganic components were calcium and phosphorus (1.7% and 1.5% respectively, by weight). Carbon dioxide (about 1% by weight) was liberated from the cyst walls on acidification. 5. The cyst walls of *H. schachtii* and the potato cyst-nematode (*Heterodera rostochiensis*) contained different amounts of the same amino acids. They also differed in their inorganic content and in the nature of the hexosamine present.

The beet cyst-nematode (*Heterodera schachtii* Schmidt) attacks a wide range of plants in the Chenopodiaceae and Cruciferae and is economically important wherever sugar beet is grown. It resembles the potato cyst-nematode (*Heterodera rostochiensis*) in many ways, but *H. rostochiensis* attacks a different range of plants and differs also in its response to hatching agents, in its resistance to desiccation and in having a diapause. Some of the differences may be related to the composition of the structural materials of the nematodes. This paper describes the composition of the cyst wall of *H. schachtii*, and compares it with that of *H. rostochiensis* (Clarke, 1968).

EXPERIMENTAL

Materials. *H. schachtii* cyst walls were isolated by the method used for *H. rostochiensis* (Clarke, 1968) and, when dried *in vacuo* over P_2O_5, yielded about 15mg/1000 cysts. The methods used to determine N, P, amino acids, carbohydrate, uronic acid, polyphenol and lipid were as described by Clarke (1968).

Hexosamine. A modified Elson–Morgan method (Allison & Smith, 1965) was used to determine hexosamine in the hydrolysate obtained by treating the cyst walls for 24h with 6M-HCl in sealed tubes at 100°C. The hexosamine present in samples of the hydrolysate evaporated to dryness *in vacuo* over KOH was separated from most other components by ion-exchange column chromatography (Gardell, 1953; Crumpton, 1959). Samples of the column fractions were used to determine

hexosamine with 2,4,6-trinitrobenzenesulphonic acid (Kelleher & Smith, 1968). The fractions containing hexosamine were combined, concentrated by evaporation and dried *in vacuo* over KOH. The purified material was used for ninhydrin degradation (Stoffyn & Jeanloz, 1954) and paper chromatography.

Paper chromatography. Whatman no. 1 paper was used and developed by downward elution with one of the following solvent systems: (*a*) pyridine–ethyl acetate–water–acetic acid (5:5:3:1, by vol.); (*b*) butan-1-ol–ethanol–water (4:1:1, by vol.). The detecting agents (Elson–Morgan and aniline–phosphoric acid reagents) were prepared and used as described by Smith (1960).

Ash. Ash was determined by heating the cyst walls at 500°C until the weight was constant. Similar determinations were made with cyst walls previously dialysed against water or 0.01M-HCl for 3 days, or extracted with 5% EDTA (sodium salt) (adjusted to pH7.2 with 5M-NaOH) for 5 days and then washed with water. Elements present in the ash were detected by spectrographic methods. Calcium was determined by flame photometry, and aluminium by a colorimetric method.

Carbon dioxide. The CO_2 liberated from cyst walls on acidification was measured by the direct method (Dixon, 1943) in a Warburg apparatus at 25°C. The CO_2 evolution was measured over 1h.

RESULTS

The quantitative values in the following sections are the average of at least three concordant determinations.

Protein and amino acids. The dried cyst walls

Table 1. *Composition of cyst walls of* H. schachtii *and* H. rostochiensis

	Composition (%)	
	H. schachtii	H. rostochiensis (Clarke, 1968)
Protein (N corrected for glucosamine N × 6.25)	68	72
Lipid	6	2
Hexosamine	3.3	1.5
Carbohydrate	2	0.5
Polyphenol	2	2
Ash	17	5

contained 11.2% nitrogen. On hydrolysis of the cyst walls with 6M-hydrochloric acid in a sealed tube at 100°C, 93% dissolved. The dark-brown insoluble material contained about 2% nitrogen. The hydrolysate was dissolved in 0.1 mM-norleucine in 0.1 M-hydrochloric acid. Samples (1.0 ml) containing about 400 μg dry wt. of hydrolysate and 13.1 μg of norleucine/ml were used for amino acid analyses with a Technicon AutoAnalyzer and gave the amounts of amino acids in the hydrolysate shown in Table 2. The recovery from the column was 95% as measured by the nitrogen content (micro-Kjeldahl) of the hydrolysate added to the column, and the total calculated nitrogen content of the amino acids was eluted.

Hexosamine. Hexosamine was slowly liberated when cyst walls were hydrolysed with 6M-hydrochloric acid for 6–36h. The hexosamine (3.3%) was liberated from the cyst walls after 24h hydrolysis. The hexosamine in the hydrolysate was eluted as a single band on an ion-exchange column (Crumpton, 1959), with the same peak eluent volume (74ml) as an authentic sample of galactosamine. Glucosamine had a peak eluent volume of 62ml. Paper chromatography of the purified hexosamine and of samples of galactosamine, and glucosamine [solvent system (a), detecting agent Elson–Morgan reagent] gave spots with R_F values of 0.28, 0.28, and 0.33 respectively. Chromatography of the ninhydrin degradation products [solvent system (b), detecting agent aniline–phosphoric acid] obtained from the purified hexosamine, and from galactosamine, and glucosamine gave spots with R_F values of 0.24, 0.24, and 0.19 respectively.

Carbohydrate and uronic acid. Cyst-wall hydrolysates contained 2% sugars and a trace of uronic acid (<0.05%).

Polyphenols. The phenol content of cyst-wall hydrolysates was 2% (calculated as catechol).

Lipid. A mixture of chloroform–methanol (1:1, v/v) extracted 6% of soluble material from the cyst walls.

Table 2. *Amino acid composition of hydrolysates of the cyst walls of* H. rostochiensis *and* H. schachtii

The amino acid contents of the cyst walls are ranges of values (% by wt. of total amino acids) obtained from analyses of three batches of cyst walls; the total protein content, calculated from the N content (Kjeldahl) less hexosamine N × 6.25, was 68%. Data for *H. rostochiensis* are from Clarke (1968).

	Amino acid composition after 24h hydrolysis (% by wt. of total amino acids)	
Amino acid	H. schachtii	H. rostochiensis
Glu	17.0–17.8	7.8– 8.4
Gly	16.7–16.8	17.9–18.5
Pro	10.8–11.5	18.6–19.0
Hyp	8.0– 8.7	5.4– 5.8
Asp	6.9– 7.5	5.7– 7.1
Ala	6.0– 6.4	12.9–13.8
Cys	4.5– 6.4	5.7– 7.3
Ser	4.7– 4.9	3.4– 5.4
Lys	3.3– 3.7	1.8– 2.8
Thr	3.1– 3.5	1.9– 2.9
Met	2.6– 3.3	2.4– 3.1
Arg	2.9– 3.1	1.5– 2.0
Val	2.0– 2.4	1.7– 1.9
Leu	1.7– 2.1	1.5– 3.6
Phe	1.4– 1.8	0.8– 1.5
NH₃	1.3– 1.6	1.1– 1.3
His	1.2– 1.4	1.4– 1.7
Tyr	1.1– 1.3	0.6– 1.3
Ile	1.1– 1.2	0.7– 1.0
Orn	Trace	Trace

Ash. The dried cyst walls gave 17% ash. Spectrographic analysis showed the presence and the orders of magnitude of the following elements: Al, Ca > 1%; Fe, Mg, 1%; Si < 1%; Cu, Mn, Na, 5000p.p.m.; Ti, 1000p.p.m.; Zn < 1000p.p.m.; Ba, Sr, V, Ni, Pb, 500p.p.m.; Sr, 300 p.p.m.; Sn, 100p.p.m.; Mo, 50p.p.m.; Cr, Co, <50p.p.m. The calcium content of the ash was about 10% as measured by flame photometry; the aluminium content was ≤ 1%.

The ash content of the cyst walls was not changed by dialysis against water, but was decreased by dialysis against 0.01 M-hydrochloric acid (ash 10%) and largely eliminated by extraction with EDTA (ash 1%).

Phosphorus. The phosphorus content of the cyst walls was 1.5%.

DISCUSSION

Lee (1966) and von Brand (1966) have reviewed the composition of nematode cuticle including some information about *Heterodera* spp. The cyst wall of *Heterodera* spp. is formed from the body wall of

the female nematode when it dies. Table 1 compares the gross composition of the cyst walls of *H. schachtii* and of *H. rostochiensis* (Clarke, 1968). The major component of the cyst walls of *H. schachtii* and *H. rostochiensis* was protein. Table 2 compares the amino acid composition of acid hydrolysates of the cyst walls. Proline was a major amino acid in the hydrolysates, which also contained hydroxyproline. The relative amounts of some of the amino acids differed appreciably. Glutamic acid was more abundant, and proline and alanine less abundant, in the cyst walls of *H. schachtii*. Cystine was obtained from the cyst walls of both species. McBride & Harrington (1967) found *Ascaris* cuticle protein contained cystine, which was involved in the cross-linkage of the collagen molecules. The amino acid analyses indicate collagen-like protein in *H. schachtii* cyst walls of different composition from that of *H. rostochiensis* cyst walls. Analyses of hydrolysates of the cuticle of second-stage larvae of *H. schachtii* suggested it had an amino acid composition similar to that of the cyst wall. Differences in the protein composition of suitable tissues such as cuticle, or cyst walls, might be useful in distinguishing related species of nematodes.

The protein of the cyst walls of *H. schachtii* was tanned, as indicated by their dark colour, the presence of material insoluble on acid hydrolysis and of polyphenols in the hydrolysates. *H. schachtii* cyst walls contained somewhat more hexosamine, carbohydrate and lipid than do those of *H. rostochiensis*. The hexosamine obtained in hydrolysates of *H. schachtii* cyst walls was identified as galactosamine by paper and column chromatography, whereas the hexosamine present in *H. rostochiensis* cyst-wall hydrolysates was glucosamine (Clarke, 1968).

The inorganic content of the cyst walls of *H. schachtii* and *H. rostochiensis* differed appreciably. *H. schachtii* cyst walls contained 17% ash, but *H. rostochiensis* cyst walls only 5%. The bulk of the inorganic material in *H. schachtii* cyst walls was bound by ionic and van der Waals forces, because extraction with EDTA decreased the amount of ash to about 1%. However, the binding forces were strong enough to prevent loss of inorganic material when the cyst walls were dialysed against water (ash content unchanged), and for much inorganic material to be retained (ash 10%) when cyst walls were dialysed against 0.01M-hydrochloric acid.

Spectrographic analysis of the ash from *H. schachtii* cyst walls indicated the presence of small amounts of various elements and greater amounts of calcium and aluminium. Quantitative determinations showed the ash contained about 10% calcium and ≼1% aluminium. The cyst walls contained an appreciable amount of phosphorus (1.5% P), and some carbonate (the CO_2 evolved on acidification was about 1% by weight of the cyst walls). The Ca/P molar ratio was about 0.9 [$Ca_3(PO_4)_2$ requires a Ca/P ratio 1.5]. von Brand (1966) reported that the amount of inorganic material in parasitic trematodes, nematodes and Acanthocephala ranged between 0.6 and 1.1% of the fresh tissue, but much larger values, up to 41% of the dry matter or 19.2% of the fresh tissues, have been found for cestodes. Much of the inorganic content of cestodes is in the form of calcareous corpuscles deposited throughout the tissue. Light and electron microscopy (Wieser, 1953; Günther & Kämpfe, 1966) has not revealed the presence of similar corpuscles in *H. schachtii* cyst walls, so the inorganic material is distributed more uniformly throughout the cyst wall. Mineralization of collagen and mucoprotein skeletal materials of animals is well known, but the present work seems to be the first indicating mineralization of tissue of a member of the Nematoda. The cyst walls of *H. schachtii* and *H. rostochiensis* were obtained from cysts produced on different host plants (sugar beet and potato, respectively) grown in different soils. It is therefore possible that the amount of inorganic material found in the cyst walls is affected by differences in the environment, e.g. soil composition or mineral content of the host plant.

Whether the inorganic content of the body wall of the adult female of *H. schachtii* is similar to that of the cyst wall is unknown. However, the relationship of the two materials makes this possible, if no barriers exist to prevent ion penetration of the living tissue. Differences between the structural materials of *Heterodera* species may be responsible for differences in response to hatching agents, in addition to differences in resistance to desiccation and in the tendency to diapause. The composition of the cyst wall is relevant to the process of hatching, not only because of its immediate role as a casing for the eggs, but also because its composition may be closely related to that of the body wall of the larvae. Recent work, e.g. by Doncaster & Shepherd (1967), supports the view that hatching agents act directly on the larva within the egg, i.e. act at the larval body wall or within. The principal component of the cyst wall, and probably also of the larval body wall, is collagen-like protein (possibly in the form of glycoprotein). The ion-binding properties of collagens are well known. Some of the ion species bound can produce extensive physical changes in the collagen. For example, experiments with calf skin collagen (Weinstock, King & Wuthier, 1967) showed that Sr^{2+}, Ca^{2+} and Mg^{2+}, in amounts as small as 1 μequiv./g of collagen or less, were particularly effective in preventing the aggregation of tropocollagen into fibrils, and in causing the disaggregation of fibrillar collagen. Changes in the

physical structure of the larval body-wall tissues produced by bound ions could be responsible for the changes in permeability suggested by Dropkin, Martin & Johnson (1958) as a possible mode of action of hatching agents. The extent to which the unhatched larvae bind ions is not known. However the capacity of the larval body wall to bind ions and the susceptibility of the species to hatching by particular ions may both be reflected in the inorganic composition of the body-wall tissues at subsequent stages of development, and in that of the cyst walls. There was more total inorganic material and calcium in the cyst walls of $H. schachtii$ (ash 17%, Ca^{2+} content about 1.7%, as percentage by weight of cyst walls) than in the cyst walls of $H. rostochiensis$ (ash 5%, Ca^{2+} content about 0.05%, as percentage by weight of cyst walls). Clarke & Shepherd (1966) found 21 inorganic ions that were moderately or very active as hatching agents for $H. schachtii$, but only ten were moderately or very active hatching agents for $H. rostochiensis$. Ca^{2+} ions were moderately active as a hatching agent for $H. schachtii$, but were nearly inactive for $H. rostochiensis$.

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