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The Chemical Composition of the Cyst Wall of the Potato Cyst-Nematode, *Heterodera rostochiensis*

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1. Cyst walls of the potato cyst-nematode (*Heterodera rostochiensis* Woll.) were isolated by sieving a suspension of crushed cysts. About 12mg. of dried cyst walls was obtained from 1000 cysts. 2. The cyst walls contained mainly protein (72%, calculated from nitrogen content). On acid hydrolysis about 77% of the cyst wall went into solution. Of 19 amino acids present, proline, glycine, and alanine were the most abundant, and made up about 50% by weight of the total amino acids. The amino acid composition suggested that collagen-like proteins predominated in the cyst wall and larval cuticle. 3. A small amount of glucosamine (1·5%) was present in the hydrolysates, but chitin was not detected in the cyst walls. 4. Other components of the cyst walls were lipid (2%), carbohydrate (0·5%) and a small amount of inorganic matter (ash, 5%). Polyphenols (2% by wt. of the cyst walls) occurred in the acid hydrolysates. The dark pigments of the cyst wall were not indole-containing melanins.

Cysts of plant-parasitic nematodes of the genus *Heterodera* are lemon-shaped or spherical, dark-brown and about 0·3–0·8mm. in diameter. Each cyst is formed from the body wall of the female of the species. After invading a root, the larvae become sedentary on reaching a feeding site; those destined to become female enlarge greatly, rupture the root cortex but remain attached with their heads embedded, and continue to feed. The body wall of the female is translucent at first, but, once fertilized and full of eggs, the female dies and the former body wall becomes first light and finally dark brown. The yellow or 'golden' stage of the swollen females of some races of the potato cyst-nematode, *Heterodera rostochiensis*, refers to the colour of the body contents. The colour changes in the body wall of the female are accompanied by hardening and tanning, which result in a protective cyst enclosing several hundred eggs.

Lee (1966) reviewed the structure and composition of helminth cuticles including those of *Heterodera* spp. Light microscopy and electron microscopy showed that the cyst walls and larval cuticle of *Heterodera* larvae consists of several layers, some fibrous, but little is known of their chemical composition. Doliwa (1956) thought both the larval cuticle and the cyst wall were made of scleroprotein without chitin. Ellenby (1946, 1963) found evidence of a polyphenol oxidase and of polyphenols and 'masked' polyphenols in white swollen females of *H. rostochiensis*, and Kaul (1962) found a non-hydrolysable condensed catechin in cyst walls.

The structure of the cyst wall of *Heterodera* spp. is of interest because it is a protective envelope formed from the cuticle of the female when she dies, inside which the quiescent eggs survive for many years in the soil. Both hatching stimulants and toxic chemicals must penetrate the cyst wall, the egg shell and possibly also the larval cuticle to affect the larvae. There is also evidence that the cyst wall or some of its components may directly affect the hatching of the eggs (Kaul, 1962; Shepherd & Cox, 1967). The present paper reports the gross chemical composition of the cyst wall of *H. rostochiensis*.

MATERIALS AND METHODS

H. rostochiensis *cyst walls*. The dark-brown cysts were washed from the soil and separated by hand from all debris. Agitation with a mechanical stirrer of a suspension in water of the crushed cysts released the eggs. The suspension was poured on to a 61μ (100meshes/in.) sieve, which retained the cyst-wall fragments but let eggs and larvae pass through. The cyst-wall fragments were thoroughly washed on the sieve and then transferred to a small glass dish and dried *in vacuo* over P_2O_5. About 12mg. of dried cyst walls was obtained from 1000 cysts.

Nitrogen. The total nitrogen in the cyst walls was estimated by a micro-Kjeldahl procedure (Mann, 1961).

Phosphorus. The phosphorus present in cyst walls was determined by the method of Holden & Pirie (1955).

Amino acids. The cyst walls were heated with 6N-HCl in a sealed tube at 100° for 24hr., after which time the hydrolysates were filtered and dried *in vacuo* over KOH. The amounts of amino acids in hydrolysates were measured with a Technicon Auto-Analyzer (Clarke, Cox & Shepherd, 1967). The proline in the hydrolysates was also determined separately by the method of Chinard (1952), and hydroxyproline by the method of Neuman & Logan (1950) as modified by Grunbaum & Glick (1956).

Hexosamine. A modified Elson–Morgan method (Allison & Smith, 1965) was used to estimate hexosamine in the hydrolysate obtained by treating the cyst walls for 6–28hr. with 6N-HCl in sealed tubes at 100°. The hexosamine present in the hydrolysates was separated from other components by ion-exchange chromatography (Glowacka, Kopaz-Jodczyk & Popowicz, 1967). The purified material was used for ninhydrin degradation (Stoffyn & Jeanloz, 1954) and paper chromatography.

Paper chromatography. Whatman no. 1 paper was used for the chromatograms, which were developed by downward elution with 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.); (*c*) butan-1-ol–acetic acid–water (12:3:5, by vol.), (*d*) ethanol–aq. NH_3 (sp.gr. 0·880)–water (20:1:4, by vol.).

Carbohydrates. The total reducing and non-reducing saccharides in the cyst walls were determined after 2hr. hydrolysis at 100° with 1·5N-H_2SO_4 by the method of Dubois, Gilles, Hamilton, Rebers & Smith (1951).

Uronic acid. The uronic acid content of cyst-wall hydrolysates after treatment with 1·5N-H_2SO_4 for 2hr. at 100° was estimated by the method of Dische (1947).

Polyphenols. The polyphenol content of samples of cyst-wall hydrolysates after treatment with 6N-HCl for 24hr. at 100° was determined after removal of the HCl by drying *in vacuo* over KOH, by the method of Pro (1952) with a modified Folin–Denis reagent.

Lipid. The lipid content of the dried cyst walls was estimated from the weight of material obtained by successive extractions for periods of 24hr. with chloroform–methanol (1:1, v/v) at room temperature.

Ash. Ash was determined by heating the cyst walls at 500° to constant weight. Elements present in the residue were detected by spectrographic methods.

EXPERIMENTAL AND RESULTS

Protein and amino acids. The dried cyst walls contained 11·5% of nitrogen. After 24hr. hydrolysis with 6N-hydrochloric acid, 77·4% of the cyst walls dissolved. The cyst-wall hydrolysates were dissolved in 0·1mM-norleucine in 0·1N-hydrochloric acid. Samples (1·0ml.) each containing about 200μg. of hydrolysate and 13·1μg. of norleucine/ml. were added to the column of the Technicon Auto-Analyzer. Table 1 lists the amounts of amino acids detected in the hydrolysate. The nitrogen content of a 1·0ml. sample calculated from the amounts of the compounds eluted from the column was 21·8μg. The nitrogen content of a 1·0ml. sample from the same hydrolysate determined by the micro-Kjeldahl method was 22·8μg., so 96% of the nitrogen was recovered from the column.

Table 1. *Amino acid composition of hydrolysates of the cyst wall and of the egg shells of* H. rostochiensis

The total amino acid contents of cyst walls are given as 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) × 6·25, was 72%. The data for egg shells are from Clarke *et al.* (1967).

Amino acid	Amino acid composition (% by wt. of total amino acids) after 24hr. hydrolysis: Cyst walls	Egg shells
Pro	18·6–19·0	38·3
Gly	17·9–18·5	8·5
Ala	12·9–13·8	2·3
Glu	7·8–8·4	6·0
CyS	5·7–7·3	—
Asp	5·7–7·1	10·9
Hyp	5·4–5·8	5·2
Ser	3·4–5·4	7·4
Met	2·4–3·1	2·6
Leu	1·5–3·6	1·6
Thr	1·9–2·9	2·7
Lys	1·8–2·8	2·8
Arg	1·5–2·0	1·7
Val	1·7–1·9	1·9
His	1·4–1·7	2·3
NH_3	1·1–1·3	0·8
Phe	0·8–1·5	1·5
Tyr	0·6–1·3	2·1
Ile	0·7–1·0	1·4
Orn	Trace	—

Non-hydrolysable material. Hydrolysis of the cyst walls with 6N-hydrochloric acid for 24hr. at 100° left a very dark-brown material that did not dissolve on further treatment under the same conditions. The solid, which contained 2·8% of nitrogen, was collected by filtration, washed with water and dried *in vacuo* over phosphorus pentoxide. Washing the solid with 1N-sodium hydroxide removed a small amount of dark-brown soluble material. The alkali-washed solid was used for alkali degradation and for permanganate oxidation. The very dark-brown material (50mg.) was heated with sodium hydroxide (240mg.) and sodium dithionite (40mg.) in a crucible for 15min. by the procedure used by Nicolaus, Piattelli & Fattorusso (1964) for authentic melanins. The contents of the crucible were washed into a small flask. Acetic acid (2ml.) was added, and the mixture was continuously extracted with ether for 24hr. Evaporation of the ether solution *in vacuo* left a brown gum (10mg.). A sample (50mg.) of the non-hydrolysable material suspended in 1M-potassium carbonate

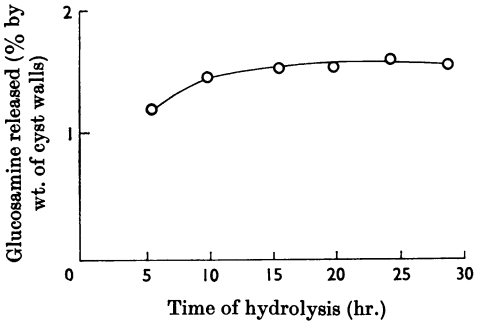

Fig. 1. Liberation of glucosamine from cyst walls by acid hydrolysis. Samples were hydrolysed in sealed tubes with 6N-HCl at 100°, for 6, 10, 16, 20, 24 and 28hr.

(0·80ml.) was oxidized with 3% (w/v) potassium permanganate (4·0ml.), and the reaction mixture was worked up as described by Piattelli, Fattorusso, Magno & Nicolaus (1962). The degradation products obtained by the above procedures were dissolved in methanol (1·0ml.). Samples (0·05ml.) were applied to paper chromatograms. The chromatograms, developed with solvent systems (*c*) and (*d*), were examined under u.v. light and sprayed with diazotized sulphanilic acid followed by 2N-sodium hydroxide, or dipped in ferric chloride–potassium ferricyanide reagent (Smith, 1960, p. 324). No red spot was obtained with the diazo reagent, but the chromatograms of the alkali-degradation products showed several fluorescent spots, some of which reacted with diazotized sulphanilic acid or the ferric chloride–ferricyanide reagent or both. Control spots of pyrrolecarboxylic acids gave cherry-red spots with the diazo reagent.

Hexosamine. Fig. 1 shows the amounts of hexosamine liberated on hydrolysis of the cyst walls with 6N-hydrochloric acid in sealed tubes at 100° for 6–28hr. The hexosamine was liberated slowly, presumably because of the resistance to hydrolysis conferred by the extensive tanning of the cyst wall. The cyst walls contained 1·5% of hexosamine, shown by 24hr. hydrolysis. The amino sugar in cyst-wall hydrolysates was isolated by ion-exchange chromatography, and was examined by paper chromatography, both of the compound itself in solvent system (*a*) with 0·2% ninhydrin in acetone as detecting agent, and of its ninhydrin-degradation products in solvent system (*b*), with silver nitrate reagent as detecting agent (Smith, 1960, p. 252). In both systems the amino sugar gave results identical with those of an authentic sample of glucosamine. The cyst walls did not give a positive van Wisselingh test for chitin.

Carbohydrate and uronic acids. Cyst-wall hydrolysates contained only 0·5% of sugar, and a trace of uronic acid (< 0·05%).

Polyphenols. The cyst-wall hydrolysates contained 2% of polyphenols (calculated as percentage catechol content of cyst walls). Extracts of the cyst walls with 70% ethanol also contained polyphenols.

Lipid. Chloroform–methanol extraction removed 2% of soluble material from the cyst walls.

Ash. The dried cyst walls gave an ash of 5% of their initial weight. Spectrographic analysis showed the presence of the following elements: Al, Ca, Fe, Si, > 1%; Zn, 3000p.p.m., Cu, Mg, Na, > 1000p.p.m.; Ba, Mn, Pb, Sr, Ti, 1000p.p.m.; Cr, Ni, Sn, V, Zn, 100p.p.m.; Co, Mo, 10p.p.m.; K, Li, present.

Phosphorus. The phosphorus content of the cyst walls was 0·2%.

DISCUSSION

Lee (1966) concluded from a survey of mainly qualitative studies of the composition of nematode cuticle that it was composed of secreted collagen associated with hyaluronic acid, chondroitin sulphate-containing acid mucopolysaccharide and a small amount of lipid. My results show that the composition of the cyst wall of *H. rostochiensis* was: protein (nitrogen content × 6·25), 72%; non-hydrolysed material, 23%; polyphenols, 2%; lipids, 2%; hexosamine, 1·5%; carbohydrate, 0·5%; ash, 5%.

Doliwa (1956) reported that cuticles of the white swollen females of *H. rostochiensis* were largely unaffected by treatment with proteolytic enzymes. However, the large protein content of the cyst wall indicated by my nitrogen analyses was confirmed by determination of the amounts of the individual amino acids in acid hydrolysates. Table 1 lists the amounts of the 19 amino acids detected. The principal amino acids, proline, glycine, alanine, and glutamic acid, made up about 58% by weight of the total amino acids. Hydroxyproline was also present. The amino acid composition suggests that collagens predominate in the cyst wall and cuticle of *H. rostochiensis*. Amino acid analyses of whole *Ascaris* cuticle (Savel, 1955*a*,*b*; Bird, 1957; Watson & Silvester, 1959) showed proline to be the most abundant amino acid, with glycine, arginine and lysine next in amount. McBride & Harrington (1967*a*,*b*) studied the unusual composition (proline 29%, hydroxyproline 2%, glycine 28%) and properties of collagen isolated from *Ascaris* cuticle in more detail. The amino acid composition of *H. rostochiensis* cyst wall suggests that a similar collagen may be present in the untanned cuticle of the living females and probably of the larvae of this species. Table 1 compares the amino acid composition of *H. rostochiensis* cyst-wall hydrolysates with that of the hydrolysates of the egg shells of this species (Clarke *et al.* 1967). The hydrolysates showed some distinct quantitative differences in the amino

acids present, although the composition of each suggested that collagen predominated in the parent material.

Hydrolysis of the cyst walls left an insoluble material, 23% by weight of the original cyst walls, containing 2·8% of nitrogen. This residue may be assumed to include polymeric material linked by non-amide bonds. To obtain evidence on the composition of this material it was further degraded by permanganate-oxidation and by alkali-fusion procedures (Nicolaus *et al.* 1964), and paper chromatograms of the reaction products were examined for the presence of indole or pyrrole derivatives. None was detected, but various unidentified phenols and polyphenols showed up on the chromatograms. Typical animal melanins contain 5·1–8·7% of nitrogen (Nicolaus *et al.* 1964) and give dihydroxyindoles and pyrrolecarboxylic acids on alkaline degradation and pyrrolecarboxylic acids on permanganate oxidation. The pigments of the cyst walls are therefore unlikely to be indole-containing melanins. The presence of phenols in the soluble and insoluble fractions obtained on acid hydrolysis of the cyst walls suggests that the tanning of the protein of the female cuticle involves these or related phenolic compounds. Ellenby (1946) found a polyphenol oxidase in the cuticle of white swollen females and evidence of the presence of polyphenols and masked polyphenols.

The amount of lipid extracted from the cyst walls was only 2%. Plant-parasitic nematode species (Krusberg, 1967) may contain 10–40% of lipid, which is thought to be used as a food reserve.

Hydrolysis of the cyst walls gave only 1·5% of amino sugar. This hexosamine was identified as glucosamine. The cyst walls gave a negative van Wisselingh test, so the glucosamine may not be present as chitin. The egg shells of *H. rostochiensis* (Clarke *et al.* 1967) contained chitin and gave 7% of glucosamine on hydrolysis.

Only 0·5% of carbohydrate was detected in the cyst-wall hydrolysates, and only a trace of uronic acid (< 0·05%). Simmonds (1958) reported substantial amounts of polysaccharide in the cast cuticle of the fourth-stage larva of *Nippostrongylus brasiliensis*, and Anya (1966) observed hyaluronic acid and chondroitin sulphate-containing mucopolysaccharide in the outer cortex of *Aspicularis tetraptera*.

The cyst wall gave an ash of 5% by weight of the original cyst wall. Mineralization, which sometimes occurs with collagen tissues, was thus not important. Spectrographic analysis of the ash showed the presence of various elements including > 1% of Al, Ca, Fe and Si. The phosphorus content of the cyst walls was only 0·2%, and the spectrum of alkali-hydrolysates of the cyst walls did not show the sharp absorption maxima of the nucleic acids.

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