Cell-wall Proteins of *Aspergillus niger* and *Chaetomium globosum*

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SUMMARY

Cell-wall fractions were obtained from *Aspergillus niger* and *Chaetomium globosum*. Non-structural protein was removed by successive washes in urea, NH_4OH and formic acid. Amino acid analyses indicated that structural wall proteins were acidic. Aspartic acid was the free amino terminal residue of the protein in both species. Comparison of the 'maps' of peptic peptides indicate that there may be some homology between the wall proteins of the two fungi.

INTRODUCTION

The status of proteins associated with the walls of fungi has been discussed in several recent reviews (Northcote, 1963; Aronson, 1965; Bartnicki-Garcia, 1968). The linkage between carbohydrate and protein components of the wall has been investigated in baker's yeast. Northcote & Horne (1952) identified a protein + mannan complex, and Falcone & Nickerson (1956) reported an additional protein + glucan complex. Eddy (1958) postulated that wall protein served to link the insoluble glucan matrix with alkali-soluble mannan and glucan. Sentandreu & Northcote (1968) identified two types of carbohydrate + amino acid linkages in a yeast cell-wall glycopeptide: *O*-mannosyl linkages to serine and threonine joined low molecular weight sugars to the peptide. Linkage to high molecular weight carbohydrate was between aspartic acid and glucosamine. There is little information about the structure of the wall protein. Numerous qualitative (e.g. Crook & Johnston, 1962; Ruiz-Herrera, 1967) and quantitative (e.g. Kessler & Nickerson, 1959; Dyke, 1964; Shah & Knight, 1968) amino acid analyses have been reported for yeasts and filamentous fungi. Korn & Northcote (1960) identified glycine and alanine as amino terminal residues, and Nickerson & Falcone (1956) showed the presence of disulphide bridges in yeast wall protein. The present work reports partial characterization of the wall proteins from two filamentous fungi, *Aspergillus niger* and *Chaetomium globosum*.

METHODS

Organisms. *Aspergillus niger* (University of British Columbia collection no. 872) and *Chaetomium globosum* (University of British Columbia collection no. 207) were grown in liquid culture medium containing (g./l.) malt extract 15.0, yeast extract 0.5, Difco Soytone 2.5 (R. J. Bandoni, personal communication). Three-week cultures were harvested by filtration on a Buchner funnel and washed with distilled water.

Preparation of cell walls. Washed mycelium was dispersed by homogenization in a Waring blender at low speed for 5 min. and broken with glass beads (200 μ diameter)

by shaking for 2 min. in water in a Braun MSK homogenizer. The fragmentation was repeated until no whole cells were visible when the preparation was examined with the light microscope. The homogenate was decanted, the beads washed several times with small volumes of distilled water, and the homogenate and washings pooled. The preparation was centrifuged at 700 *g* for 30 sec. and the supernatant fluid discarded. The pellet was suspended in water and shaken with glass beads in the disintegrator for 1 min. The wall fragments were recovered by centrifugation at 700 *g* for 30 sec. The washing procedure was repeated until the supernatant fluid was clear after centrifugation. The walls were then washed in M-NaCl and washing repeated until the supernatant fluid was clear. The preparation was purified further by two washes in 8 M-urea, one extraction in 8 M-urea for 16 hr at 4°, and two washes in 8 M-urea. After each wash, the wall fragments were recovered by centrifugation at 16,000 *g* for 5 min. The pellet was finally washed 5 times with each of: water, M-NH₄OH, water, 5 % (v/v) formic acid in water, water. The walls were dispersed thoroughly in the liquid and then centrifuged at 16,000 *g* for 5 min. The final material was lyophilized and stored at -10°. Walls purified in this way were free from cytoplasmic contamination when examined by light and electron microscopy.

Protein determination was by the method of Lowry, Rosebrough, Farr & Randall (1951) as modified by Eggstein & Kreutz (1955). Protein removed from the walls during purification was determined by using the lyophilized pooled material from each washing step. Urea and sodium chloride were removed by dialysis against running tap water for 48 hr before lyophilization.

Amino acid analysis. Dried-wall preparation (5 to 10 mg.) was hydrolysed *in vacuo* with 5.7 N-HCl (0.1 ml./mg.) for 20 hr at 110° and dried in a vacuum desiccator over KOH pellets. Quantitative amino acid analysis was done by the method of Piez & Morris (1960) with a Bio-Cal automatic amino acid analyser.

Identification of the free amino groups of wall protein. The method of Gray & Hartley (1963) was modified. Dry walls (5 mg.) were suspended in 0.2 ml. 0.1 M-NaHCO₃ and 0.1 ml. 1-dimethylaminonaphthalene-5-sulphonyl-(DNS-) chloride in acetone (0.1 %, w/v) was added. The mixture was kept at room temperature for 3 hr, dried in a vacuum desiccator and hydrolysed with 5.7 N-HCl for 6 hr at 110°. DNS-amino acids were identified by chromatography on polyamide sheets (Woods & Wang, 1967).

Hydrolysis of wall protein with pepsin. Wall material (40 to 100 mg.) was suspended in 5 % (v/v) formic acid with 4 to 10 mg. pepsin (Calbiochem. Ltd.) for 24 hr at 25°. The insoluble material was removed by centrifugation.

Peptic peptide maps. Peptides were separated by high voltage paper electrophoresis in an apparatus similar to that described by Michl (1951). The buffer systems at pH 6.5 and 2.0 were those of Ambler (1963). Complete peptide maps were obtained only after two procedures. Basic and acidic peptides were resolved by electrophoresis in the first dimension at pH 6.5 for 50 min. Resolution of neutral peptides required electrophoresis in this dimension for 180 min. Marker strips were sewn on a new sheet and second dimension electrophoresis for all peptides was at pH 2.0 for 40 min. Cystine bridges were detected by the diagonal procedure of Brown & Hartley (1963). Peptides were located by dipping papers in cadmium + ninhydrin reagent (Heilmann, Barollier & Watzke, 1957). Maps were tested for tryptophan-containing peptides by spraying with acid Ehrlich reagent (Reddi & Kodicek, 1953).

RESULTS

Preparation of walls. Complete fragmentation of mycelia was achieved after 2 or 3 homogenizations in water. After 5 water washes only traces of cytoplasm were visible by phase contrast microscopy. Some wall material was lost during washes with NaCl and urea. Less than 2 % of the dry weight of the material dissolved by washing was protein, except after formic acid treatment when protein was 5 to 10 % of the total removed. The final wall preparation from *Aspergillus niger* contained 1.76 % N, and from *Chaetomium globosum* 3.21 % N.

Table 1. Quantitative amino acid composition of fungal wall hydrolysates

Amino acid	<i>Aspergillus niger</i>		<i>Chaetomium globosum</i>	
	$\mu\text{g. amino acid}$ residue/mg. dry wt	$\mu\text{mole amino}$ acid/1000 μmole	$\mu\text{g. amino acid}$ residue/mg. dry wt	$\mu\text{mole amino}$ acid/1000 μmole
Aspartic acid	1.18	89.5	5.74	104.2
Threonine*	1.27	109.9	6.77	139.8
Serine*	1.92	192.8	3.84	92.0
Glutamic acid	0.88	59.5	7.53	121.7
Proline	0.68	65.4	4.62	99.3
Glycine	0.72	110.3	2.68	98.0
Alanine	0.76	92.7	2.89	85.0
1/2 Cystine	0.35	29.8	0.83	16.9
Valine	0.55	48.3	2.58	54.3
Methionine	0.34	22.7	0.43	6.9
Isoleucine	0.26	20.4	1.39	25.7
Leucine	0.46	25.3	3.25	60.0
Tyrosine	0.54	28.8	1.70	21.8
Phenylalanine	0.52	31.1	1.87	26.5
Lysine	0.66	44.9	0.62	10.0
Histidine	Trace	Trace	Trace	Trace
Arginine	0.40	22.5	2.82	37.7
Total	11.49	—	49.56	—

* 6 % correction applied for loss of threonine and serine during hydrolysis.

Amino acid analyses of walls. These are given in Table 1. The totals show 1.15 % protein in *Aspergillus niger* and 4.96 % in *Chaetomium globosum* walls. Both proteins contained large total amounts of threonine and serine (30 and 23 %, respectively). They were also rich in acidic amino acids (14.9 and 22.5 %, respectively). Methionine was present in walls of both species. The predominant basic amino acid was lysine in *A. niger* and arginine in *C. globosum*. Histidine was found only in traces.

Amino acid analysis of walls after digestion with pepsin showed that 10 to 20 % of the wall protein was removed. Various amounts of all amino acids were released, except for lysine, which remained almost entirely in the walls.

Free amino groups of wall protein. The DNS-derivatives of aspartic acid and $\epsilon\text{-NH}_2$ lysine were the only fluorescent products after 'dansylation' and hydrolysis of the walls of both species.

Peptide maps. Maps of the peptides released by digestion of cell walls with pepsin are shown in Fig. 1 and 2. In *Aspergillus niger* (Fig. 1a, 2a) 6 basic, 37 acidic, and 25 neutral ninhydrin-positive spots were located. In *Chaetomium globosum* (Fig. 1b, 2b)

Fig. 1. Peptide map of a peptic digest of cell-wall protein of (a) *Aspergillus niger*, (b) *Chaetomium globosum*. Electrophoresis was at pH 6.5 in the first dimension (horizontal) and at pH 2.0 in the second dimension (vertical). The dashed line marks the origin. Mobilities are relative to aspartic acid = -1.0 at pH 6.5, and +1.0 at pH 2.0. Cross-hatching indicates peptides of identical mobilities from each species. Peptide Y is the cystine-containing peptide common to both species.

Fig. 2. Map of 'neutral' peptides of a peptic digest of cell-wall protein of (a) *Aspergillus niger*, (b) *Chaetomium globosum*. The unresolved peptides (Fig. 1) having mobilities approximating to 0 at pH 6.5, were resolved by electrophoresis for 180 min. at pH 6.5 in the first dimension and at pH 2.0 in the second dimension. The dashed line marks the origin. Second dimension mobilities are relative to aspartic acid = +1.0. Cross-hatching indicates the peptides of identical mobilities from each species. Peptide Y is the cystine-containing peptide common to both species.

there were 5 basic, 37 acidic, and 24 neutral spots. Twenty-nine spots (shaded) showed identical mobilities on maps from both species. In each species 5 pairs of peptides from cystine-containing fragments were located on diagonal maps after performic acid oxidation. Only the pairs derived from spot Y (Fig. 1, 2) had the same mobilities in both species. Spot Y and the peptides derived from it stained orange-yellow with ninhydrin. No peptides containing tryptophan were detected on any of the maps.

DISCUSSION

Chemical methods for the isolation of cell walls may remove wall constituents (Kreger, 1954). The nature of the final material will vary according to the treatments which have been used in its preparation (Rogers & Perkins, 1968). Thus, isolated wall preparations cannot sensibly be equated with the functional wall of the whole organism (Crook & Johnston, 1962). In the present work an attempt was made to prepare fungal walls free from all protein which was not an integral part of wall structure. The branched structure of many wall polymers (Setterfield & Bayley, 1961) could provide a random network in which protein molecules become entangled. The extended exposure to 8 M-urea released any protein associated with the wall fragments in this way. Washings with ammonia and formic acid were included to remove protein associated with the wall by adsorption and/or ionic linkage. Reagents which might have caused extensive hydrolysis of peptide bonds or carbohydrate+protein linkages were avoided.

The amino acid analyses given in Table 1 indicate that the wall proteins of *Aspergillus niger* and *Chaetomium globosum* may be acidic and there were many acidic peptides in the peptic digests of the walls (Fig. 1). Both proteins were rich in threonine and serine. Other workers (Kessler & Nickerson, 1959; Dyke, 1964; Chattaway, Holmes & Barlow, 1968) reported similar features in wall proteins from *Saccharomyces cerevisiae*, *Candida albicans* and *Nadsonia elongata*. By using paper chromatography, Crook & Johnston (1962) did not find methionine in walls of *A. niger*, and recorded it as possibly present in *C. globosum*. We found methionine in small amounts from both of these species. Total N and total amino acids in the walls of *C. globosum* were higher than in *A. niger*. In *C. globosum* protein N (calculated from amino acid analysis) was 23.6% of the total N, whereas in *A. niger* it represented only 10%.

The identification of glycine and alanine as amino-terminal residues in yeast wall protein led Korn & Northcote (1960) to conclude that there may be more than one polypeptide chain present. In *Aspergillus niger* and *Chaetomium globosum* aspartic acid was the only free amino-terminal residue in wall protein as determined by dansylation. It is not possible to say at present how many polypeptide chains occur in the structure of wall protein.

Comparison of peptide maps (Fig. 1, 2) suggests that there is some homology between the two wall proteins. If the protein plays a vital part in wall structure and in growth, then two regions of homology may be postulated. Similar amino acid sequences would be expected around the points of carbohydrate+protein linkage, for example the linkages through aspartic acid, threonine and serine in yeast (Sentandreu & Northcote, 1968). Other regions of homology may occur around points of growth control. Nickerson & Falcone (1956) postulated that fungal wall extension occurred by the action of a protein disulphide reductase on one or more cystine bridges. The

identification of one cystine bridge (Fig. 1 and 2, spot Y) which appears similar in both species, leads us to suggest that this is a target for the action of the disulphide reductase.

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