The Peptides of the Hyphal Wall of *Neurospora crassa*

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SUMMARY

The peptides of the hyphal wall of *Neurospora crassa* have been studied to determine their physical characteristics. We have removed these peptides from the wall by treatment with 0.5 M-NaOH and shown by DEAE cellulose chromatography that there are five major fractions present. Amino acid analysis showed distinct differences in primary structure between these fractions but similarities in the proportions of hydrophilic residues and the ratios of basic residues to acidic residues of each fraction. Two unknown compounds, one acidic and the other basic, were also detected.

Extraction of the wall with NH_4OH released a high molecular weight glycopeptide complex, from which the peptides were released with NaOH. *O*-Glycosyl serine linkages were demonstrated to occur within this complex.

Enzyme digestion of either the chitin or the β -1,3-polymer of the hyphal wall failed to release peptide material. This, together with the above, suggests that the glycopeptide of the hyphal wall is a highly ordered structure of peptides joined by a branched carbohydrate linker of unknown composition, and that it is not demonstrably covalently linked to the other major components of the wall. It probably occurs *in vivo* as an extremely large structure embedded in the other polymers of the hyphal wall.

INTRODUCTION

The hyphal wall of the fungi has received considerable study as it is this portion of the organism which determines morphology and growth patterns. The chemical composition of the walls of many fungi has been studied (for review, see Bartnicki-Garcia, 1968) in an attempt to determine if alterations in this structure may lead to specific alterations in morphology. Considerable attention has been paid to the structure of the carbohydrate elements of the wall, but little has been given to other components. This is not unexpected, as polysaccharides make up 80 to 90 % of the hyphal wall, the remainder being lipid and protein. This has resulted in a paucity of information concerning the role these minor constituents play in determining morphology.

Since the first report of proteinaceous material in the wall of yeast (Eddy, 1958), there have been several reports of the presence of amino acids as a product of hydrolysis of the cell walls of various fungi. Suomalainen and his co-workers (Nurminen, Oura & Suomalainen, 1970) have described enzymatic proteins which are associated with the wall of yeast, and Northcote and co-workers have shown the presence of glycopeptide components on the wall of baker's yeast (Korn & Northcote, 1960; Sentandreu & Northcote, 1968).

The presence of a protein component of the hyphal wall of *Neurospora crassa* was established by de Terra & Tatum (1963) and Mahadevan & Tatum (1965). The latter showed the presence of amino acids in acid hydrolysates of the whole hyphal wall and of their Fraction I, which is the first base-soluble fraction of the wall. Manocha & Colvin (1967) have reported the presence of proteinaceous material in the wall of this organism which could be

liberated by digestion of the polysaccharide portions of the wall with hydrolytic enzymes. They concluded, on the basis of electron microscopy, that this protein material forms a discrete layer within the wall, and that it also lines pores within the wall. Hunsley & Burnett (1970) have also studied the wall of *N. crassa* by enzymatic digestion of various components, followed by examination by electron microscopy. They support the contention that the protein exists as one of a series of concentric layers which make up the cell wall. Enzymatic proteins have also been found to be associated with the hyphal wall of *N. crassa* (Eberhart, 1961; Mahadevan & Mahadkar, 1970; Trevithick & Metznerberg, 1966*a*), and their location (Chang & Trevithick, 1972*b*), relationship to the wall (Chang & Trevithick, 1972*a*) and function (Mahadevan & Mahadkar, 1970) have been described.

Two classes of wall-associated proteins thus exist. First, those of enzymatic function which are not associated with the wall structure *per se*, but are 'in transit' to the external medium (Trevithick & Metznerberg, 1966*a*) or trapped within the structure of the growing wall (Chang & Trevithick, 1972*a*). The second class is that described above as participating in determining the morphology of the wall itself (Hunsley & Burnett, 1970; Manocha & Colvin, 1967). No work has been reported concerning the physical characteristics, such as subunit structure and molecular weight, of this component of the fungal cell wall. This report describes the initial experiments towards establishing the structure of this interesting component of the hyphal wall of *Neurospora crassa*.

METHODS

Growth of organisms and preparation of hyphal wall. *Neurospora crassa*, wild-type strain RL 3-8A from the Rockefeller University, was grown in 15 l carboys with Vogel's (1964) Medium N using 2 % sucrose as the carbon source. The carboys were inoculated with mycelia sheared in a Waring Blender. Mycelium was harvested on filter paper, washed thoroughly with distilled water and lyophilized. The dried material was ground in a mortar and stored at 4 °C. Hyphal walls were prepared by a modification of the method of Mahadevan & Tatum (1965). The ground mycelium was suspended in 2 % sodium lauroyl sarcosinate (1 g mycelia/100 ml) and stirred in the cold overnight. After thorough washing with distilled water by centrifugation, the suspension of hyphal walls was dialysed overnight at 4 °C against distilled water and lyophilized. The walls were found to be free of cytoplasmic contamination by phase-contrast microscopy.

Strain H605-1-3A (serine-1, F.G.S.C. no. 116), a serine auxotroph, was grown on liquid Vogel's Medium N supplemented with 0.1 mg serine and 0.5 mg alanine/ml, together with 6.25 µC of uniformly labelled [¹⁴C]serine (Schwartz-Mann, Orangeburg, New York, U.S.A.). Harvesting and preparation of hyphal wall were as above.

Preparation of hyphal wall peptides. Hyphal wall was suspended (10 mg/ml) in 0.5 M-NaOH and stirred at 4 °C overnight. The suspension was filtered through glass-fibre filter paper under vacuum and two volumes of absolute ethanol added to the filtrate. After 2 h at 4 °C, the resulting precipitate, which contained a polygalactosamine polymer (Fraction I of Mahadevan & Tatum, 1965), was removed by filtration and the filtrate brought to pH 7.6 with HCl. The ethanol was removed by rotary evaporation under vacuum. The peptides were desalted by passing them through a column of Bio-Gel P-2 (Bio-Rad Laboratories, Richmond, California, U.S.A.), with 0.2 M-NH₄OH as the eluant. The peptide-containing fractions, identified by absorbance at 280 nm, were combined and the NH₄OH removed by rotary evaporation under vacuum.

Peptide material was also released from the wall by treatment with 25 % NH₄OH for 3 h at 37 °C with stirring. The suspension was filtered, the NH₄OH removed from the filtrate

by rotary vacuum evaporation and the resulting solution chromatographed on a column of Bio-Gel P-150, 50 to 100 mesh. Material removed from the wall with NH_4OH was also subjected to gel-filtration chromatography on a column of Bio-Gel A 0.5 M in 0.1 M- NH_4HCO_3 (pH 9.0) with 6 M-urea. The sample was incubated in the same buffer at 37 °C for 3 h before application to the column. The protein-containing fractions were identified by absorbance at 257 and 280 nm.

Acid extraction of the hyphal wall was performed by suspending the wall (10 mg/ml) in 1 N-HCl and stirring the suspension for 10 min at room temperature. The residue was separated from the extract by filtration as before and the acid removed by lyophilization. The remaining material was dissolved in distilled water made slightly basic by the addition of NH_4OH .

Quantitation. Estimation of protein concentration was attempted using both the Biuret method (Gornall, Bardawill & David, 1949) and that of Lowry, Rosebrough, Farr & Randall (1951). The response curve given by hyphal wall peptide with both these tests was not linear over a concentration range in which bovine serum albumin gave a linear response, and so these methods of estimation were not used. Basic hydrolysis (see below) was found to produce a linear response and so was used to measure the protein present. The sample was dissolved in 1 ml of 1.0 M-NaOH, placed in a polyethylene centrifuge tube and 0.1 ml of 10 M-NaOH added. The solution was heated for 3 h at 130 °C, cooled and brought to pH 5.5 with acetic acid. The amino acids present were measured with sodium propionate-ninhydrin reagent (Rosen, Berard & Levenson, 1962) against a standard of leucine. Molecular weights of 3800 for the peptides and 100 as the average amino acid were assumed in order to translate the quantity of amino acids present to mg of protein.

Column chromatography. The peptides were separated on a 2 × 20 cm column of DEAE cellulose (Schleicher & Schuell, Keene, New Hampshire, U.S.A.) equilibrated with 0.001 M-tris-HCl, pH 7.4. The sample was applied and washed on the column with the same buffer. The chromatogram was developed with a 600 ml linear gradient, with 0.001 M-tris-HCl, pH 7.4, as the starting buffer and 0.5 M-NaCl in 0.001 M-tris-HCl, pH 7.4, as the final buffer. The effluent was monitored at 280 nm and 10 ml fractions were collected.

DEAE cellulose chromatography was also performed in the presence of urea, to ascertain if the peptides eluted from the standard DEAE cellulose columns were stable aggregates. The conditions (column size, buffer and elution gradient) were the same as above, except that 6 M-urea (Ultra-pure, Schwarz-Mann, Orangeburg, New York, U.S.A.) was incorporated into the buffers. The peptide sample, either whole wall extract or DEAE cellulose fractions, was dissolved in tris-urea and incubated for 3 h at 37 °C in a water bath. This material was then applied to the column and the chromatogram developed.

Gel filtration to determine molecular weights was performed on a column of Bio-Gel P-60, 100 to 200 mesh. The column dimensions were 1.5 × 80 cm. The eluant was 0.1 M-tris-HCl, pH 7.4, containing 8 M-urea and 0.5 M-NaCl. One-ml fractions were collected and analysed by absorbance at 210 nm. Calibration standards were obtained by cyanogen bromide cleavage of horse-heart cytochrome *c* and sperm-whale myoglobin (Sigma Chemical Co., St Louis, Missouri, U.S.A.) according to the method of Gross (1967).

Amino-acid analysis. The peptide mixture or fractions were freed of salt as described above and hydrolysed for 22 h with 6 N-HCl at 105 °C in evacuated tubes. The HCl was removed by rotary evaporation. Amino acids were separated and measured with a Durrum Amino Acid Analyser (Durrum Chemical Corp., Palo Alto, California, U.S.A.) after the method of Spackmann, Stein & Moore (1958). Durrum pico buffers were used for analysis.

Identification of linkage amino acid. Strain H605 was grown and hyphal walls prepared as described. A portion of the wall was suspended in a solution containing 0.25 M-NaOH and 0.3 M-NaBH₄ (Ventron, Beverly, Massachusetts, U.S.A.) and stirred overnight at 4 °C. The suspension was filtered, ethanol added and the suspension filtered again. Excess NaBH₄ was removed by acidification and addition of methyl alcohol, followed by flash evaporation. The remaining material was passed through a Bio-Gel P-2 column and the peptide-containing fractions were pooled and reduced in volume by flash evaporation. A portion of this radioactive peptide was hydrolysed with 6 N-HCl. The hydrolysate was chromatographed on cellulose thin-layer chromatography plates ('Mannogram', Schwarz-Mann, Orangeburg, New York, U.S.A.). The solvents used were: 1, *n*-butanol:acetic acid:water (12:3:5, by vol.); 2, phenol:ammonia (100:1, v/v); and 3, *t*-butanol:water:methyl ethyl ketone:diethylamine (80:80:40:8). Appropriate standards were run on outside channels of the same plate to identify the amino acids. Location was by spraying these outer channels with 1 % ninhydrin in *n*-butanol. The unstained areas were collected in disposable pipettes by vacuum and the amino acids eluted from the cellulose with 0.01 M-acetic acid. Solvent systems 1 or 2 were used to separate alanine from serine and glycine. The serine-glycine mixture, which was not separated by solvent 1, was concentrated and rerun in solvent 3, which separates these two amino acids. The amino acids were eluted from the collected cellulose into 20 ml scintillation vials and 15 ml of Aquasol (New England Nuclear, Boston, Massachusetts, U.S.A.) added to each vial. Determination of radioactivity was accomplished in a liquid scintillation counter.

Radioactive hyphal wall peptide, isolated from walls by treatment with 0.5 N-NaOH or 1.0 N-HCl, was also treated as described to determine the radioactivity in serine and alanine.

Hyphal wall digestion by enzymes. A 100 mg portion of hyphal wall was suspended in 30 ml sodium acetate buffer, 0.005 M, pH 6.0, together with 10 mg chitinase (EC. 3.2.1.14, Schwarz-Mann, Orangeburg, New York, U.S.A.) and incubated at 37 °C in a shaking water bath. After 48 h an additional 10 mg of the enzyme was added and the digestion continued for 24 h. The hyphal wall was then collected and washed three times with distilled water by centrifugation. A portion of the digested wall was analysed for the presence of glucosamine by acid hydrolysis followed by thin-layer chromatography. The remainder of the wall was treated with 0.5 N-NaOH and the filtrate analysed for released protein material by basic hydrolysis.

The above procedure was repeated with Glusulase (Endo Laboratories, Garden City, New York, U.S.A.) which digests the β -1,3-carbohydrate polymer. The buffer was 0.01 M-K₂HPO₄ plus 0.003 M-citric acid, pH 6.0, and 1 ml of enzyme solution was added at each time period. Controls were hyphal walls suspended in buffer without enzymes and treated exactly as described for the enzyme digested walls.

RESULTS

Release of peptides from the hyphal wall. Portions of wall were suspended in 0.5 M-NaOH and incubated at 25 °C for varying periods of time to ascertain the time course of release of the peptides. Samples were filtered and the filtrate subjected to basic hydrolysis and ninhydrin quantitation. The kinetics of release are shown in Fig. 1. Approximately 50 % of the peptides were released during the first 30 min of treatment, with complete release being observed after 6 h. This showed the extreme lability to strong base of the linkages which hold the peptides within the hyphal wall. Gel filtration of the material released by NaOH showed that this material was exclusively of low molecular weight.

Fig. 1. Release of peptide material from hyphal wall with 0.5 N-NaOH at 25 °C. Each point is the average of duplicate determinations from each of three experiments.

Table 1. *Peptide material released from the hyphal wall by different extraction methods and comparison of acid and basic hydrolysis methods for measurement*

Data given as percentage by weight of the hyphal wall.

NaOH treatment		Hydrolysis	
Time (h)	Temperature °C	Acid*	Alkali†
6	25	8.23	7.59
18	4	8.42	7.50

* Summation of individual amino acids after automatic amino acid analysis.

† Estimation by manual ninhydrin method.

To determine the most favourable method for the release of the peptides from the hyphal wall, portions were suspended in 0.5 M-NaOH and treated for 6 h at 25 °C or 18 h at 4 °C. The amount of released peptides was determined by either basic hydrolysis with ninhydrin quantitation or acidic hydrolysis followed by automatic amino-acid analysis. The data in Table 1 show good agreement between the two methods of analysis and also show that the longer period of base treatment in the cold released an amount of peptide equivalent to that released at the higher temperature. The cold treatment was used in order to avoid possible degradation of the peptide by the base at the higher temperatures.

Table 2 shows the amino acids produced by acid hydrolysis of the peptide material released from the wall by treatment at 4 °C for 18 h in 0.5 M-NaOH.

Treatment of the wall with 25 % NH_4OH at 37 °C for 3 h also released much of the peptide, although there was considerable variation in the amount released – typically, between 60 and 75 %. Gel filtration showed that this material consisted of a disperse, high molecular weight peak, with an average weight of approximately 100000 daltons. Gel filtration in the presence of urea established that this material was not an aggregate held together by non-covalent linkages. On a column of Bio Gel A 0.5 M in 6 M-urea, the NH_4OH -released material gave a peak similar to that obtained by gel filtration without urea. A portion of the NH_4OH extract, treated with 0.5 N-NaOH overnight at 4 °C and then made to 6 M with

Table 2. *Amino-acid composition of material removed from the hyphal wall by treatment with 0.5 N-NaOH*

Amino acid	Percentage of total micromoles	Amino acid	Percentage of total micromoles
Asp	12.43	Met	1.21
Thr	8.18	Ile	3.26
Ser	8.64	Leu	6.85
Glu	10.24	Tyr	2.91
Pro	0.72	Phe	2.25
Gly	13.36	Lys	6.43
Ala	10.85	His	2.26
$\frac{1}{2}$ Cys	0.71	Arg	3.67
Val	5.82

Fig. 2. DEAE-cellulose chromatography of hyphal wall peptides. Column dimensions were 2×20 cm and the buffer used 0.001 M-tris-HCl, pH 7.4. A linear gradient of NaCl was used for elution. Ten-ml fractions were collected. Numbered bars represent the fractions which were pooled to form the peptide fractions I to V.

urea, gave a single low molecular weight peak with the same elution volume as the peptides released from the hyphal wall by direct NaOH treatment.

Strong acid (1.0 N-HCl) was also effective in removing peptide from the wall. This material was found to be similar to that removed from the wall by NaOH, as co-chromatography on sieving the gel showed no difference in the elution pattern of the peptides separated from the wall by these two methods.

Separation and amino-acid composition of the released peptides. The hyphal wall peptides were prepared by 0.5 M-NaOH treatment of the wall, desalted and applied to a column of DEAE cellulose. The column was developed as described. A typical elution pattern is given in Fig. 2, showing the five major components that were separated. They are referred to as fractions I to V.

The elution pattern shown in Fig. 2 has been confirmed, both for elution position and the relative proportion of the fractions, by many repetitions with samples taken from different wall preparations and from different growths of mycelia. Further, fractions which were separated by DEAE cellulose chromatography and then rerun in the presence of urea exhibited no difference in elution profiles. This showed that the peptides obtained by base

Table 3. Amino acid composition of DEAE fractions I to V

Values for each amino acid shown as percentage of total micromoles of amino acids present in each sample.

Amino acid	Fraction no.				
	I	II	III	IV	V
Asp	15.13	14.64	14.04	15.00	17.30
Thr	8.19	7.44	7.13	7.50	6.62
Ser	8.73	8.14	7.52	6.78	6.22
Glu	10.51	10.84	11.22	11.15	13.02
Gly	11.23	13.70	14.72	12.38	11.80
Ala	13.54	12.80	11.30	11.96	11.17
Cys	0.70	—	0.78	—	—
Val	8.70	7.56	7.02	7.15	6.05
Met	0.42	0.90	0.85	0.39	0.30
Ile	4.00	3.66	3.60	4.00	4.58
Leu	6.18	6.56	6.66	6.57	6.56
Tyr	1.70	3.19	3.62	4.00	2.97
Phe	3.92	4.57	5.04	5.54	6.51
His	1.52	1.41	1.57	1.71	0.70
Lys	4.02	2.73	2.58	2.73	0.77
Arg	0.72	1.17	1.51	2.19	4.80
Pro	0.70	0.60	0.72	0.86	0.54
Calculated molecular weight *	14 125	17 366	20 797	18 840	7016
Basic residue to acid residue ratio†	0.244	0.208	0.224	0.253	0.206
Unknown I‡	949	763	503	None	2381
Unknown II‡	79	52	47	405	1507

* Based on arginine content.

† Sum of histidine, lysine and arginine μ moles/sum of glutamic acid and aspartic acid μ moles.

‡ Given as area of response curve/total μ moles of amino acids in sample.

treatment of the hyphal wall and separated by DEAE chromatography are normal constituents of the wall, do not change in composition owing to age differences of the mycelia or methods of extraction, and are not artefacts of aggregation caused by interaction of the peptides.

Material obtained by NaOH treatment of the high molecular weight fraction removed from the wall by 25 % NH_4OH or of peptides removed from the wall with acid, was also chromatographed on DEAE cellulose. The elution pattern was the same as that of peptides obtained by direct treatment of the wall with NaOH. Co-chromatography of the separated fractions from all types of preparations revealed identical elution behaviour.

Fractions I to V were concentrated, desalted and subjected to acid hydrolysis. The amino acids present were determined by automatic analysis. The results of three separate preparations, given as the average values for each amino acid, are shown in Table 3. Determination of tryptophan was not performed. The ratios of micromoles of basic (lysine, histidine and arginine) to acidic (glutamic and aspartic acids) residues are also given for each fraction. Although the total acidic residues varied between the separated fractions, the ratio remained fairly constant.

Amino-acid analysis also showed the presence of two unidentified compounds, one (unknown I) having an acidic and the other (unknown II) a basic character. These components were present in considerable quantities, but no further attempts have been made

Fig. 3. Estimation of molecular weights of the hyphal wall peptides by gel filtration in the presence of urea. Calibration standards are cyanogen bromide fragments of myoglobin (myoglobin I, II and III) and cytochrome *c* (cytochrome I and II). The location of the elution position of the hyphal wall peptides is indicated.

to identify them. However, as the unknown I was absent in fraction IV, and the quantities present in the other fractions were significant, these unknown components warrant further study.

Molecular weight of the peptides. Molecular weights of the separated fractions were determined by gel filtration in the presence of urea and high salt. Under low salt conditions, these peptides bind to the gel, with an increase in the volume needed to elute the fractions from the column. The results of the high-salt chromatography are shown in Fig. 3, with the distribution coefficient (K_d) of the standards and the unknown plotted against the log of the molecular weights. The K_d was calculated by the following formula:

$$K_d = \frac{V_t - V_e}{V_t - V_o},$$

where V_t is the elution volume of tritiated water, V_o the elution volume of bovine serum albumin and haemoglobin (which elute at the void volume of the column), and V_e the elution volume of the molecule in question. Each point on the calibration line represents the average of three separate determinations. There was no difference in the elution volumes of either standard or peptide fractions when they were run separately or together. The estimated molecular weight for all peptide fractions was 3800 daltons. A mixture of peptides produced a single, symmetrical peak with a K_d of 0.607. Thus by this technique there was no detectable difference between the molecular weights of any of the fractions which could be separated by DEAE cellulose chromatography.

A comparison of the molecular weight estimated by gel filtration and the molecular weight estimated from amino acid analysis shows a considerable discrepancy. As shown in Table 3, the estimated molecular weights of the fractions ranged from about 7000 to 20000 daltons. Dialysis membranes which retain cytochrome *c* in tris-HCl buffer at pH 7.4 would not

Fig. 4. Release of peptides from material extracted from the cell wall by NH_4OH . (a) (Run 1) Gel filtration on P-150 column of untreated NH_4OH extract. Fractions five to fifteen were pooled; (Run 2) gel filtration on same column of pooled material from (1) after 80 min in 0.5 M-NaOH at room temperature. (b) Increase in absorbance at 241 nm of pooled material during treatment with 0.5 N-NaOH.

retain the hyphal wall peptides, either as a mixture or as separate fractions. It would thus appear that the lower value obtained by gel filtration is the correct one and that the values obtained from the amino-acid analysis are the result of some degree of heterogeneity within the separated fractions. Attempts to resolve the peptide fractions further were not successful.

Linkage of the peptides. The lability of the linkages of the wall peptide to alkali is indicative of the presence of covalent bonds between a sugar and either serine or threonine (*O*-glycosyl serine or *O*-glycosyl threonine) (Neuberger, Gottschalk & Marshall, 1966). To exclude the possibility of an ester bond, which is also base-labile, hyphal wall was suspended in tetrahydrofuran with 0.3 M- LiBH_4 and boiled under reflux for 10 h. If the bonds holding the peptides and carbohydrates together were of the ester type, this procedure should have released them (Gottschalk & Murphy, 1961). However, 92 % (average of three experiments) of the peptide remained with the sedimentable residue after this treatment. This provides evidence for the release of the peptides by a typical β -elimination reaction.

NaOH treatment of the high molecular weight peptide material removed from the hyphal

Table 4. *Radioactivity of alanine and serine recovered by thin-layer chromatography after acid hydrolysis of hyphal wall peptide released by different methods*

Method of peptide release	C.p.m. applied to chromatogram	C.p.m. recovered	Percentage of total c.p.m. applied
Acid (1.0 N-HCl)	570	—	—
Alanine		51	8.9
Serine		187	32.8
Total		238	41.7
Base (0.5 N-NaOH)	802	—	—
Alanine		53	6.8
Serine		65	8.0
Total		118	14.8
Base (0.25 N-NaOH + 0.3 M-NaBH ₄)	640	—	—
Alanine		120	18.8
Serine		55	8.6
Total		175	27.4

wall with NH₄OH provided evidence for the existence of *O*-glycosyl serine within this complex. A solution of the material extracted by NH₄OH was placed on a column of Bio-Gel P-150 and the high molecular weight peak pooled and concentrated. This was made to 0.5 M-NaOH and a portion taken for absorbance measurements. After 80 min, the remainder was again run on the P-150 column. The results are depicted in Fig. 4. The increase in absorbance at 241 nm was caused by the production of dehydroserine (Carubelli, Bhavanandan & Gottschalk, 1965), which is a product of the base-catalysed β -elimination reaction that breaks the *O*-glycosyl serine bond. This was accompanied by the appearance of a low molecular weight fraction from the gel-filtration column. If this reaction was continued, the low molecular peak was enriched at the expense of the high molecular weight fraction until the latter disappeared. From these results, we concluded that the material extracted from the wall by NH₄OH is actually small peptides joined to a branched carbohydrate linker by *O*-glycosyl serine bonds, which forms a much larger molecule *in vivo*.

Acid treatment of the walls released low molecular weight peptide material which was similar in behaviour to that released by treatment with NaOH. If acid-extracted material was dissolved in 0.5 N-NaOH and absorbance of the solution at 241 nm recorded, there was no change over a period of 3 h. This showed that the released material, although similar to that removed from the wall by strong base treatment, was no longer capable of undergoing a β -elimination reaction. Therefore, the release of the peptides by acid probably resulted from an attack on the sugar moiety of the *O*-glycosyl amino-acid linkage. This is in accord with the findings of Sentandreu & Northcote (1968) concerning the acid-sensitivity of these linkages in yeast cell-wall glycopeptide.

If *O*-glycosyl serine bonds are present between the peptide and carbohydrate, treatment with base followed by acid hydrolysis should demonstrate a loss of the serine molecules in this linkage. These linkage molecules can be recovered by reduction of the peptide with NaBH₄ before acid hydrolysis (Tanaka, Bertolini & Pigman, 1964). This will produce alanine by hydrogenation of dehydroserine, which is formed by the β -elimination reaction which cleaves the *O*-glycosyl serine bond. If radioactive serine is incorporated into the cell wall peptides and the walls treated with NaBH₄ in base, an increase in the radioactivity of alanine and a decrease in that of serine should be observed. The results of such an experiment are shown in Table 4. The peptide of a serine-requiring strain was labelled with

[^{14}C]serine. Peptides were removed from the wall with either NaOH, NaOH + NaBH₄, or HCl. They were then hydrolysed with HCl and the resulting amino acids separated by thin-layer chromatography. The radioactivity of the alanine and serine was then determined. Extraction by acid was used as the control as it was assumed that destruction of both serine and alanine was minimal with this treatment. Treatment with base alone showed a 75 % reduction in labelled serine, with a small loss of alanine. Upon reduction with NaBH₄ the same loss of serine was observed, but with a 147 % increase in alanine. This conversion of serine to alanine by base treatment and reduction gave further evidence for the existence of *O*-glycosyl serine linkages between the peptides and a carbohydrate linker.

Digestion of the wall with chitinase and glusulase gave evidence as to the location of the peptide in the hyphal wall. After digestion with chitinase the residue was found to be free of glucosamine. However, at least 85 % of the peptide components remained associated with the residue as compared to the buffer-treated controls. Measurement of peptide material remaining with the hyphal wall residue after digestion with glusulase is difficult, as this enzyme preparation is known to contain a protease (Holden & Tracey, 1950). However, a considerable amount of low molecular weight peptide material was released by treatment of the digested residue with NaOH. These results showed that although peptide material comprised a portion of the hyphal wall of this organism, there was no demonstrable covalent attachment of this component to other major constituents of the wall.

DISCUSSION

The results of this investigation partially confirm and extend the observations of previous workers concerning the occurrence of protein in the hyphal wall of *Neurospora crassa*. Our data on the amino-acid composition of the peptides released from the wall are similar to the data on the amino acids of hydrolysed whole wall reported by Livingston (1969). However, they are substantially different from those of Mahadevan & Tatum (1965) and Manocha & Colvin (1967). In the former, separation and identification was by paper chromatography and only seven amino acids were found. We have found all normally occurring protein amino acids to be present, with the exception of hydroxyproline, whose absence has been noted before (Manocha & Colvin, 1967). Manocha & Colvin (1967) found an absence of valine and only trace amounts of arginine. We have found these amino acids to be present in significant amounts – 5.8 and 3.6 % of total moles respectively. They also reported higher (approx. 5 %) values for both glutamic and aspartic acids than shown by the present work. The data reported here also give a minimum value for the protein content of the hyphal wall of *N. crassa* – 8.63 % by weight. This is less than reported previously (de Terra & Tatum, 1963; Manocha & Colvin, 1967) on the basis of whole wall hydrolysis. However, there are some free amino acids remaining on the wall residue after base treatment (C. R. Wrathall, unpublished results) which could give rise to an overestimate of protein by such hydrolysis, as well as differences in the amino acids recovered from such treatment.

The finding of significant amounts of peptide material in the residue after enzymatic digestion of the chitin or β -1,3-carbohydrate polymer of the walls, also confirms earlier work (Manocha & Colvin, 1967; Hunsley & Burnett, 1970). The hypothesis of Hunsley & Burnett (1970), that the peptide component of the wall is part of a glycoprotein reticulum with no demonstrable covalent attachment to the rest of the wall, is supported by our results. Our experiments, in which the high molecular weight complex was extracted from the hyphal walls by NH₄OH and small peptides released from this complex by subsequent NaOH-catalysed β -elimination reactions, support their hypothesis. The initial extraction with

ammonia may have been strong enough to cleave a few bonds, solubilizing the complex, while the second treatment with stronger base was needed to effect the complete destruction of the carbohydrate-peptide linkages.

Acid extraction of the hyphal wall also released peptide material which was similar in its molecular weight and DEAE cellulose elution profile to that removed by NaOH but which did not give, on treatment with NaOH, the increase in absorbance at 241 nm which is characteristic of β -elimination involving *O*-glycosyl serine. The acid treatment probably destroyed the sugar which was involved in the bond between the carbohydrate and the peptide and released the latter (Sentandreu & Northcote, 1968). The peptides that were removed from the hyphal wall by NaOH or HCl therefore appear to have been released from the reticulum itself, rather than freed by the breaking of bonds which attach them to the rest of the wall.

The structure of the glycopeptide reticulum of the wall, in light of the above, is of considerable interest. First, the carbohydrate linker must be a highly branched polymer with a number of reducing ends, as the *O*-glycosyl serine linkage involves the anomeric carbon of the carbohydrate. Since the usual carbohydrate branching utilizes rather than generates anomeric carbons, the branching of this linker must be of an unusual nature. A molecule such as this, with phosphate links between the carbohydrate chains, has been suggested to occur in the galactomannan-peptide of the yeast *Cladosporium wernecki* (Lloyd, 1970). Secondly, the fact that the separation by DEAE cellulose chromatography of the peptides extracted from the hyphal wall by both acid and base treatment is highly repeatable indicates that the structure of the reticulum is closely controlled. The possibility that heterogeneity arises through a random destruction of the terminal residues of the peptides caused by extraction methods is eliminated by this repeatable separation, unless destruction occurs at specific locations in each molecule. The molecular weights of the separated peptides, as determined by molecular-sieve chromatography, are very similar. This finding also indicates a stringent control of the structure of the glycopeptide reticulum. The heterogeneity of the separated peptides, as shown by the molecular weights calculated from the amino-acid composition, indicates that some differences in the structure of the peptides are permissible but that these must be relatively minor.

Amino acid analysis of the separated peptides showed the acidic and hydrophilic nature of these molecules. However, the differences in quantity of some of the amino acids, in particular arginine, lysine, glutamic and aspartic acids and the unknowns I and II, show that there are distinct differences between the peptide fractions. The similarity of the basic to the acidic residue ratio for each of the fractions supports the idea that there is a general similarity in the charge distribution within the reticulum.

This investigation provides the first evidence for the subunit structure of the glycopeptide reticulum of the hyphal wall of *Neurospora crassa* and also provides data which may be of some value in speculations on the function of the peptide component of the hyphal walls. Manocha & Colvin (1967) have argued for a strictly structural function for the peptide fraction. However, other hypothesis concerning the function of the wall peptides have been proposed. Slayman & Slayman (1970) proposed that the acidic residues of the peptides of the wall may be an 'in-transit' holding point for Na⁺ and K⁺ ions before they reach the cell membrane for transport, providing an exogenous pool of these ions which is closely associated with each cell. The results here show substantial quantities of charged amino acid residues in each peptide fraction but with very similar acidic-basic residue ratios. This would support the hypothesis that the peptides acted as an ion-exchange storehouse for cations as well as anions. The quantity of ions present on the hyphal wall would depend

upon the pH of the medium in which the cells were grown but still would give the cell an immediately available source of many different ions, rather than depend upon simple diffusion of these ions through the hyphal wall.

Yabuki & Fukui (1970) have described a fraction, isolated from the hyphal walls of *Aspergillus oryzae*, which prevents the binding of α -amylase to the hyphal walls. This factor was extracted by base and behaved in a manner similar in some respects to the peptides described here. Their findings imply that there is a unique binding site on the hyphal wall for the enzyme α -amylase and that this site can be blocked by a 'masking factor'. The peptides we have obtained from the wall of *Neurospora crassa* would be excellent candidates for the binding sites for various enzymes which are known to be extracellular in this organism. The enzyme invertase, which is excreted into the medium by *Neurospora*, is known to contain hexosamine (Metzenberg, 1963). If the amino groups of this carbohydrate prosthetic group are not acetylated, the carboxyl groups of the glutamic and aspartic acids of the wall peptide would provide excellent binding sites for this enzyme. The α -amylase of *A. oryzae* is also a glycoprotein (Yabuki & Fukui, 1970) and could be bound to the wall in a similar manner. Eylar (1965) has postulated that the carbohydrate portion of glycoproteins is necessary for their excretion into the extracellular space. In the case of the fungi, one could postulate that the wall peptides would bind these enzymes until a specific substrate occurs in the medium. A particular enzyme, or group of enzymes, needed to process this substrate would then be released from the wall by a now unknown mechanism and the enzymes replaced on the wall by excretion from the cell. Preliminary experiments from this laboratory show that peptides isolated from the walls of cells grown on maltose have less of peptide fraction II than those grown on sucrose. The use of these peptides as binding sites for extracellular enzymes would help to explain the presence of protein-lined pores in the cell wall (Manocha & Colvin, 1967). The molecular sieving properties of the cell wall (Trevithick & Metzenberg, 1966*a, b*) might also be partially attributed to these binding sites on the protein lining these pores. The diversity which we have shown in the peptide fraction of the hyphal wall lends support to the argument that a physiological, as opposed to structural, role may be assigned to this wall component. The actual role that they may play in the function of the fungal cell remains to be elucidated.

The results of this investigation sustain the use of the term 'remarkable' to describe the glycoprotein component of the hyphal wall of *Neurospora crassa* by Hunsley & Burnett (1970). The results of the acid and base solubilization presented here show that this wall component is made up of numerous subunits but that the structure of the larger polymer is remarkably constant in terms of the peptides present. The fact that these peptides differ in their amino acid composition gives evidence for the necessity of retaining a specific structure and indicates that this portion of the hyphal wall is a very important one in the maintenance of normal growth in this organism.

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