The Composition of the Cell Wall of *Fusicoccum amygdali*

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1. The cell wall of *Fusicoccum amygdali* consisted of polysaccharides (85%), protein (4–6%), lipid (5%) and phosphorus (0.1%). 2. The main carbohydrate constituent was D-glucose; smaller amounts of D-glucosamine, D-galactose, D-mannose, L-rhamnose, xylose and arabinose were also identified, and 16 common amino acids were detected. 3. Chitin, which accounted for most of the cell-wall glucosamine, was isolated in an undegraded form by an enzymic method. Chitosan was not detected, but traces of glucosamine were found in alkali-soluble and water-soluble fractions. 4. Cell walls were stained dark blue by iodine and were attacked by α -amylase, with liberation of glucose, maltose and maltotriose, indicating the existence of chains of α -(1 $\rightarrow$ 4)-linked glucopyranose residues. 5. Glucose and gentiobiose were liberated from cell walls by the action of an exo- β -(1 $\rightarrow$ 3)-glucanase, giving evidence for both β -(1 $\rightarrow$ 3)- and β -(1 $\rightarrow$ 6)-glucopyranose linkages. 6. Incubation of cell walls with *Helix pomatia* digestive enzymes released glucose, N-acetyl-D-glucosamine and a non-diffusible fraction, containing most of the cell-wall galactose, mannose and rhamnose. Part of this fraction was released by incubating cell walls with Pronase; acid hydrolysis yielded galactose 6-phosphate and small amounts of mannose 6-phosphate and glucose 6-phosphate as well as other materials. Extracellular polysaccharides of a similar nature were isolated and may be formed by the action of lytic enzymes on the cell wall. 7. About 30% of the cell wall was resistant to the action of the *H. pomatia* digestive enzymes; the resistant fraction was shown to be a predominantly α -(1 $\rightarrow$ 3)-glucan. 8. Fractionation of the cell-wall complex with 1M-sodium hydroxide gave three principal glucan fractions: fraction BB had $[\alpha]_D +236^\circ$ (in 1M-sodium hydroxide) and showed two components on sedimentation analysis; fraction AA₂ had $[\alpha]_D -71^\circ$ (in 1M-sodium hydroxide) and contained predominantly β -linkages; fraction AA₁ had $[\alpha]_D +40^\circ$ (in 1M-sodium hydroxide) and may contain both α - and β -linkages.

The wilting action of *Fusicoccum amygdali* Del., the pathogen of almond and peach canker, is caused by a toxin, fusicoccin (Graniti, 1964). Some years ago Professor E. B. Chain initiated a programme to isolate sufficient fusicoccin for the study of its chemical and biological properties. Since the wild-type *F. amygdali* produced very little fusicoccin when grown in submerged culture and did not sporulate, the possibility of using protoplasts for strain selection was investigated. Although protoplasts could be released from the fungal mycelium by using *Helix pomatia* digestive enzymes, yields were low (G. T. Banks, unpublished results). The aim of the present work was to study the chemical structure of the cell wall of *F. amygdali* with a view to developing more efficient methods of protoplast formation.

MATERIALS AND METHODS

Growth of organism. *F. amygdali* Del., obtained from Dr A. Graniti (Istituto di Patologia Vegetale, University of Bari, Italy), was grown at 24°C in 400-litre stirred fermenters by Mr G. T. Banks by using a medium containing glucose (3%), NaNO₃ (0.33%), KH₂PO₄ (0.1%), KCl (0.05%), MgSO₄·7H₂O (0.05%), FeSO₄·7H₂O (0.001%) and milled soya-bean meal (0.2%). In one experiment soya bean was replaced by yeast autolysate (0.1%). Mycelium was harvested after 3.5 days (maximum growth) and washed with water.

Preparation of cell walls. Method A. *F. amygdali* mycelium (25 g wet weight) was disrupted by six passages through an X-press (Edebo, 1960; Yoshida, Heden, Cedergren & Edebo, 1961) at -25°C and a pressure of 15 tons/in². Cell walls were isolated by the method of Mendoza & Villanueva (1963) and finally freeze-dried and stored over P₂O₅.

Method B. *F. amygdali* mycelium (3kg wet wt.) was suspended in water (9 litres) at 0°C and disrupted by two passages through a Manton-Gaulin homogenizer (The A.P.V. Co. Ltd., Manor Royal, Crawley, Sussex, U.K.) at a pressure of 1000 kg/cm². The homogenate was centrifuged (1500g, 30 min) and the residue was washed three times with 0.03 M-sodium phosphate buffer, pH 7.6, containing 10g of sodium lauryl sulphate/l, and five times with water, all at 4°C, before freeze-drying or further fractionation.

Cell walls, prepared by either method, were free from unbroken cells or residual cytoplasm, as revealed by examination with an Ortholux microscope, at a magnification of $\times 1000$, either with phase-contrast (Appelgarth, 1967) or by staining with Cotton Blue (0.5g of water-soluble Aniline Blue/l of lactophenol). For all analyses cell walls, or cell-wall fractions, were dried to constant weight *in vacuo* at 60°C over P₂O₅.

I.r. spectra. These were determined by using a Unicam SP.200G spectrophotometer. Materials were enclosed in KBr discs (Barker, Bourne & Whiffen, 1960).

Optical rotations. These were measured in a 1 dm cell at 20–25°C. Values for monosaccharides were of 1% solutions in water at equilibrium and for polysaccharide fractions were of 1% solutions in 1 M-NaOH.

Paper chromatography and electrophoresis. Descending chromatography on Whatman no. 1 paper (analytical) or Whatman 3MM paper (preparative) was done with the following solvents: *A*, ethyl acetate–pyridine–water–acetic acid (5:5:3:1, by vol.); *B*, butan-1-ol–pyridine–water–benzene (5:3:3:1, by vol.); *C*, ethyl acetate–pyridine–water (36:10:15, by vol.); *D*, ethyl acetate–pyridine–water (8:2:1, by vol.); *E*, propan-1-ol–ammonia (sp.gr. 0.88)–water (6:3:1, by vol.); *F*, propan-1-ol–ethyl acetate–water (7:1:2, by vol.); *G*, butan-1-ol–pyridine–water (6:4:3, by vol.). Electrophoresis was done on Whatman 3MM paper with the following buffers: *1*, 0.05 M-sodium tetraborate, pH 9.3; *2*, 0.05 M-sodium germanate, pH 10.7. Detecting reagents were: (a) AgNO₃–NaOH (Trevelyan, Procter & Harrison, 1950); (b) aniline–hydrogen phthalate (Wilson, 1959); (c) Elson–Morgan and Morgan–Elson reagents (Partridge & Westall, 1948); (d) molybdate (Hanes & Isherwood, 1949).

Hydrolysis conditions. For amino acids heating was done in 6 M-HCl at 100°C for 24, 48 and 72 h; amino sugars were released by heating with 5.7 M-HCl at 100°C for 18 h; neutral sugar hydrolysates were prepared by stirring materials with 72% (v/v) H₂SO₄ at 0°C for 1 h, diluting them to 1 M-acid and heating them for 4 h at 100°C. Neutralization was with NaOH, Ba(OH)₂ or Amberlite IR-4B (OH[−] form).

Analytical methods. The following methods were used: total N, Crook & Johnston (1962); total P, Bartlett (1959); free and bound lipids, ash and total protein (ninhydrin method), Bartnicki-Garcia & Nickerson (1962); amino acid analyses, Thomas (1970), corrected as described by Tristram & Smith (1963); total hexose, Dubois, Gilles, Hamilton, Rebers & Smith (1956); hexosamine, Boas (1953); *N*-acetyl-D-glucosamine, Reissig, Strominger & Leloir (1955); glucose by using D-glucose oxidase (Fleming & Pegler, 1963); galactose by using D-galactose dehydrogenase (Doudoroff, Contopoulou & Burns, 1958); other neutral sugars, Wilson (1959); reducing sugars with the

Nelson–Somogyi reagent (Hodge & Hofreiter, 1962), by using glucose and gentiobiose as standards.

Gel chromatography. Aq. 0.5% solutions of polymers (0.4 ml) were passed through a column (1.4 cm $\times$ 29 cm) of Sephadex G-200, equilibrated and eluted with water with a flow rate of 4.5 ml/h. Fractions (0.05 ml) were analysed for total hexose. Sephadex G-200 and dextrans for calibration were obtained from Pharmacia (G.B.) Ltd., London W.13, U.K.

Enzymes. A freeze-dried preparation of the digestive enzymes of *H. pomatia* was obtained from Industrie Biologique Francaise, Gennevilliers, France. Hog pancreatic α -amylase (500 i.u./mg) and *Streptomyces griseus* chitinase (40 units/mg) were obtained from Worthington Biochemicals Corp., Freehold, N.J., U.S.A. Pronase was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., and had 45 PUK/mg (PUK, proteinase activity unit of Kaken; Narahashi, 1970). Potato acid phosphatase (2 units/mg) was obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. Incubations of sugar phosphates (0.1%) with the enzyme (0.01%) were done at 37°C in 0.01 M-sodium acetate buffer, pH 4.8, for 18 h.

Basidiomycete QM 806 exo- β -(1 $\rightarrow$ 3)-glucanase was prepared by the method of Reese & Mandels (1959). The crude enzyme had 9 units of β -(1 $\rightarrow$ 3)-glucanase activity/mg when assayed as described by Huotari, Nelson, Smith & Kirkwood (1968) and 1.1 units of amylase activity when assayed against soluble starch under the same conditions. For further purification a mixture of crude enzyme (2700 units) in water (8 ml) and McIlvaine's (1921) citrate–phosphate buffer, pH 3.5, (16 ml) and a 1% suspension of pachyman, [freed from starch by treatment with α -amylase (Noble & Sturgeon, 1968)] in water (20 ml) was stirred for 30 min at 0°C and then centrifuged (15000g, 10 min). The residue was washed three times by resuspension and centrifugation with the same buffer and was then resuspended in 0.05 M-sodium acetate, pH 4.8 (15 ml), and incubated at 37°C for 8 h, when most of the pachyman was hydrolysed to glucose and gentiobiose. After centrifugation (15000g, 10 min) the supernatant was dialysed against 2 mM-sodium phosphate buffer, pH 7.0, and freeze-dried. The purified enzyme [700 units of β -(1 $\rightarrow$ 3)-glucanase activity] had no action on cellulose, pustulan, pullulan, amylose, amylopectin, cellobiose or gentiobiose, when incubated with the appropriate substrate under the conditions of the β -(1 $\rightarrow$ 3)-glucanase assay.

Periodate oxidation of sugar phosphates. Samples (1 ml) of sugar phosphate fraction X and authentic samples of glucose 6-phosphate, galactose 6-phosphate and mannose 6-phosphate were mixed with 25 mM-sodium metaperiodate (4 ml) and stored in the dark at 4°C for 24 h. Redistilled ethylene glycol (0.1 ml) was added and after 5 min, 25 mM-BaCl₂ (4 ml) was added. The precipitate was removed by centrifugation and the supernatant was concentrated to 0.5 ml. The product had the same mobility as glyceraldehyde 3-phosphate (*R_F* 1.74) when chromatographed in solvent *E*.

Fractionation of cell walls. With alkali. Cell walls (200 mg) were homogenized in water (20 ml), then NaBH₄ (20 mg) was added, the pH was adjusted to 8.0 with CO₂ and the mixture was stirred for 24 h at 25°C in an atmosphere of N₂. A 20 ml sample of 2 M-KOH was added and

the mixture was stirred for 24 h at 25°C and then centrifuged (10000g, 20 min), and the residue was washed with 1M-KOH (2×20 ml). The combined supernatant and washings were neutralized with acetic acid and dialysed exhaustively against water. The resultant precipitate was centrifuged as described above and the residue was washed with water (2×20 ml) and freeze-dried to give fraction *B*, and the combined supernatant and washings were freeze-dried to give fraction *A*. The residue remaining after extraction with 1M-KOH was neutralized, dialysed against water and freeze-dried to give fraction *C*. Further extraction of this fraction with alkali did not result in significant further solubilization.

RESULTS

All results refer to cell walls prepared by method *A* unless otherwise stated.

Chemical composition. The composition of cell walls did not vary significantly from batch to batch or with the method of preparation (Table 1).

Neutral sugars. These were isolated from acid hydrolysates of cell walls by paper chromatography (solvent *A*) and had the same paper chromatographic (solvents *A*, *B* and *C*) and electrophoretic mobilities (buffers 1 and 2) as authentic specimens. Further identification was as follows. D-Glucose had $[\alpha]_D +52^\circ$; Pigman (1957) gives $[\alpha]_D +52.7^\circ$; the β -penta-acetate, prepared by the method of Fischer (1916), had m.p. and mixed m.p. of 132°C with an authentic specimen; the monosaccharide was able to react quantitatively with glucose oxidase, confirming the D-configuration (Pazur & Kleppe, 1964).

D-Galactose had $[\alpha]_D +80^\circ$; Pigman (1957) gives $+80.2^\circ$; the methylphenylhydrazone, prepared by the method of Araki (1938), had m.p. and mixed m.p. 188–189°C with an authentic specimen;

quantitative reaction of the monosaccharide with β -D-galactose dehydrogenase (Wallenfels and Kurz, 1962) confirmed that all the galactose was in the D-configuration.

D-Mannose had $[\alpha]_D +14^\circ$; Pigman (1957) gives $+14.6^\circ$.

L-Rhamnose had $[\alpha]_D +8.2^\circ$; Pigman (1957) gives $+8.9^\circ$.

No ribose or deoxyribose could be detected, indicating the absence of significant amounts of RNA or DNA.

Hexosamine. D-Glucosamine was the only hexosamine detected in acid hydrolysates of cell walls by paper chromatography (solvents *A* and *B*). It was isolated by paper chromatography as the crystalline hydrochloride, which had $[\alpha]_D +73^\circ$, the value for the authentic material being $+72^\circ$. The *N*-benzyloxycarbonyl derivative, prepared by the method of Chargaff & Bovarnick (1937), had m.p. and mixed m.p. of 214–215°C (decomp.).

Amino acids. The amino acid composition of the cell wall is given in Table 2. Methionine and proline were also detected, but were not determined quantitatively.

I.r. spectrum. This had absorption maxima at 890 and 850 cm⁻¹, characteristic of β - and α -glucans respectively (Michell, 1970).

Iodine stain. Cell walls stained dark blue with 0.1M-iodine solution, suggesting the presence of chains of α -(1→4)-glucopyranose residues. This property was unchanged when the organism was grown in a completely soluble medium in which soya-bean meal was replaced by yeast autolysate.

Characterization of cell-wall chitin. Cell-wall chitin, prepared both by the methods of Houwink & Kreger (1953) and BeMiller (1965), was found to

Table 1. *Hyphal wall composition of F. amygdali*

Component	% of cell-wall dry weight
Total anhydro hexose	79
Anhydro-D-glucose	69
Anhydro-D-galactose	3.8
Anhydro-D-mannose	2.8
Anhydro-L-rhamnose	1.3
Anhydro-xylose	1.0
Anhydro-arabinose	0.5
Anhydro-D-glucosamine	4.5
Total protein (ninhydrin method)	6.1
(total N—amino sugar nitrogen)	
×6.25	6.0
Free lipid	2.2
Bound lipid	2.7
Total N	1.34
Total P	0.10
Ash	1.2

Table 2. *Amino acid composition of F. amygdali cell walls*

The results are calculated for anhydro amino acid.

Amino acid	% of cell-wall dry weight
Ala	0.25
Arg	0.70
Asx	0.38
Glx	0.33
Gly	0.19
His	0.12
Ile	0.14
Leu	0.24
Lys	0.11
Phe	0.27
Ser	0.28
Thr	0.20
Tyr	0.20
Val	0.25

Scheme 1. Method for preparation of cell-wall chitin and determination of glucan linkage composition. Enzyme incubations were done to completion by using the conditions described in the text.

* Gentiobiose was calculated by difference of total hexose and glucose or total reducing sugars and glucose by calibrating the reagents against both glucose and gentiobiose. The trace amount of laminaribiose formed was insignificant. The two methods agreed within the limits of experimental error.

† One β-(1→3)-linkage is cleaved for each molecule of gentiobiose liberated.

contain a considerable amount of neutral carbohydrate. A product, free from neutral carbohydrate, was obtained by treatment of cell walls with α-amylase and then exo-β-(1→3)-glucanase, and extraction of the residue with sodium hydroxide (Scheme 1). The insoluble residue (4.4% of the cell-wall dry wt.) had an X-ray diffraction pattern

and i.r. spectrum almost identical with those of crustacean chitin. When a suspension of this material (1.2mg) in 0.05M-phosphate buffer, pH 6.3 (1ml), containing *Streptomyces* chitinase (0.5mg) and *H. pomatia* digestive enzymes (0.5mg), was incubated at 37°C for 24h, *N*-acetyl-D-glucosamine was liberated in 95% yield.

Chitosan could not be detected in the cell walls either by the Van Wisselingh test (Tracey, 1955) or by extraction with hydrochloric acid or acetic acid (Bartnicki-Garcia & Nickerson, 1962).

Action of enzymes on the cell walls. (a) Enzymes of *H. pomatia*. When cell walls were incubated with *H. pomatia* digestive enzymes, glucose and *N*-acetyl-D-glucosamine were liberated as revealed by chromatography of the supernatant (solvents *B* and *D*). The amounts liberated as a function of time are shown in Fig. 1. The supernatant, after a 96h incubation, was dialysed against water to remove diffusible sugars, and the dialysis residue was found to contain a polymeric fraction (8% of the cell wall), the composition of which is given in Table 3. When this fraction was passed down a column of Sephadex G-200 it was largely excluded from the gel, indicating a mol.wt. greater than 200000. Examination of 0.5M-sulphuric acid hydrolysates of the diffusate by paper chromatography revealed, in addition to glucose and glucosamine, small amounts of xylose and arabinose.

The water-insoluble residue (30% of the original cell walls), which was resistant to further action of the snail enzymes, contained glucose with small amounts of galactose, mannose, xylose, arabinose

and rhamnose and traces of glucosamine. Of this residue 80% was soluble in 1M-NaOH; this soluble fraction had $[\alpha]_D +212^\circ$, gave only a trace of a blue stain with iodine and had an i.r. spectrum, almost identical with that of an *Aspergillus niger* cell wall α -(1 $\rightarrow$ 3)-glucan, $[\alpha]_D +260^\circ$ (Fraction IV R, Johnston, 1965). This spectrum, which had absorption maxima at 850cm⁻¹ and 820cm⁻¹ is characteristic of α -(1 $\rightarrow$ 3)-glucans (Bacon, Jones, Farmer & Webley, 1968), but differs from those of amylose, amylopectin, glycogen, dextran or nigeran (Barker *et al.* 1960). Further evidence for the presence in the cell walls of a predominantly α -(1 $\rightarrow$ 3)-glucan is given in the following paper (Obaidah & Buck, 1971).

(b) Exo- β -D-(1 $\rightarrow$ 3)-glucanase. Cell walls (2mg) were incubated with basidiomycete QM806 exo- β -(1 $\rightarrow$ 3)-glucanase (2 units, Reese & Mandels, 1959) in 0.05M-sodium acetate buffer, pH 4.8, (1ml) at 50°C. After 24h, when the reaction was complete, the mixture was boiled for 5 min and centrifuged (15000g, 20min). Examination of the supernatant by paper chromatography (solvents *B*, *C*, *F* and *G*) revealed glucose and a reducing sugar with the same R_F values as gentiobiose; a trace of a component with the same R_F value as laminaribiose was also detected. This indicates the presence of β -(1 $\rightarrow$ 3)- and β -(1 $\rightarrow$ 6)-linked glucopyranose residues in the cell walls.

(c) α -Amylase. When cell walls (500mg) were incubated with hog pancreatic α -amylase (3000i.u.) in 2mM-sodium phosphate buffer, pH 6.9, containing 6.7mM-NaCl (40ml) at 37°C, they rapidly lost the ability to stain with iodine and appeared thinner and less rigid when examined microscopically. After 24h, when the reaction was complete, paper chromatography (solvents *B*, *F* and *G*) of the supernatant revealed that glucose, maltose and maltotriose were the only sugars liberated.

Treatment of intact *F. amygdali* mycelium with α -amylase under the same conditions led to rapid loss of ability to stain with iodine.

(d) Pronase. When cell walls (200mg) were incubated with Pronase (90PUK) in 0.03M-sodium phosphate buffer, pH 7.6 (20ml), containing

Fig. 1. Hydrolysis of cell walls by *H. pomatia* enzymes. Cell walls (10mg/ml) were stirred with snail-enzyme preparation (1mg/ml) in 0.01M-citrate-phosphate buffer, pH 5.8, at 37°C. At intervals samples were withdrawn, then heated at 100°C for 5 min, and cooled and centrifuged (15000g, 20min). The supernatants were assayed for total hexose ($\blacktriangle$), glucose ($\bullet$) and *N*-acetylglucosamine ($\blacksquare$).

Table 3. Composition of the non-diffusible supernatant after incubation of cell walls with snail enzymes

	% of cell-walls (as anhydro sugar)	% of cell- wall total
Glucose	0.5	0.6
Galactose	3.1	83
Mannose	2.1	76
Arabinose	0	—
Xylose	Trace	—
Rhamnose	1.2	90

2% (v/v) ethanol at 37°C, the reaction was complete after 48 h when 67% of the cell-wall protein had been removed and a water soluble, non-diffusible fraction, containing 30% of the galactose, 11% of the mannose, 17% of the rhamnose and 38% of the P originally present in the cell walls, was released. Examination of this fraction on Sephadex G-200 showed it to be heterogeneous both with respect to molecular weight and carbohydrate composition (Fig. 2).

Isolation and characterization of sugar phosphates. The non-diffusible fraction liberated from cell walls by Pronase was hydrolysed with 2M-HCl at 100°C for 75 min and sugar phosphates were isolated essentially as described by Sentandreu and Northcote (1968). The product was separated by

Fig. 2. Sephadex G-200 elution profile of the non-diffusible fraction released from cell walls on incubation with Pronase. The column void volume, determined with Blue Dextran, was 9.0 ml. The elution volume was 14.2 ml for dextran 70 (M_w 7×10^4) and 23.8 ml for dextran 20 (M_w 2×10^4). ●, Total hexose. The galactose (○), mannose (▲) and rhamnose (■) contents were determined after 1M-H₂SO₄ hydrolysis.

chromatography on Whatman 3MM paper (solvent *E*) into P₁ and two sugar phosphate fractions, X and Y, both of which reacted positively with the aniline-hydrogen phthalate and molybdate sprays. Fraction X (R_F 1.15) was shown to consist mainly of galactose 6-phosphate with small amounts of mannose 6-phosphate and glucose 6-phosphate as follows. It had the same mobility on paper chromatography (solvent *E*) as galactose 6-phosphate, mannose 6-phosphate and glucose 6-phosphate and on treatment with acid phosphatase gave rise to galactose and small amounts of mannose and glucose. Periodate oxidation of fraction X (and also authentic samples of the above three hexose 6-phosphates run in parallel) gave a product with the same mobility as glyceraldehyde 3-phosphate on paper chromatography (solvent *E*). When this product was treated with acid phosphatase and then reduced with sodium borohydride, glycerol was produced. Fraction Y (R_F 1.30), on treatment with acid phosphatase, mainly gave rise to galactose, as well as a small amount of mannose and traces of xylose and glucose. It clearly consists also of a mixture of sugar phosphates, but the positions of the phosphate groups have not been established.

Determination of the cell-wall glucan linkage composition. The method is summarized in Scheme 1 and the results for two batches of cell walls are shown in Table 4. Corrections were not made for the possibility that α -amylase or exo- β -(1 $\rightarrow$ 3)-glucanase may leave short stubs of α -(1 $\rightarrow$ 4)- or β -(1 $\rightarrow$ 3)-linkages attached to the cell-wall residue or for the small numbers of α -(1 $\rightarrow$ 4)-glucopyranose linkages present in the α -(1 $\rightarrow$ 3)-glucan fraction (Obaidah & Buck, 1971); such corrections would not have a significant effect on the values given. The α -(1 $\rightarrow$ 3)-glucan fraction had an i.r. spectrum almost identical with the α -(1 $\rightarrow$ 3)-glucan isolated from cell walls of *A. niger* (fraction IV R, Johnston, 1965), and was free from β -linkages (no absorption maximum at 890 cm⁻¹).

Fractionation of the cell walls. (a) With ethylenediamine. Cell walls were extracted with ethylenediamine and further separated on the basis of their water solubility as described by Korn & Northcote

Table 4. Approximate glucan linkage composition of cell walls

Linkage	% of total glucose residues	
	Batch 1 (prepared by method A)	Batch 2 (prepared by method B)
β -(1 $\rightarrow$ 3)-Glucopyranose	51	48
β -(1 $\rightarrow$ 6)-Glucopyranose	10	8
α -(1 $\rightarrow$ 3)-Glucopyranose	29	33
α -(1 $\rightarrow$ 4)-Glucopyranose	10	11

(1960) for yeast cell walls. The amounts and compositions of the fractions obtained are given in Table 5. All the fractions stained dark blue with iodine and had absorption maxima at 850cm^{-1} and 890cm^{-1} ; however, the peak at 890cm^{-1} was more intense in fraction *EA*, which also had a low optical rotation, suggesting that it contained a larger amount of β -glucan.

(b) With chloral hydrate. About 10% of the cell walls was soluble in aq. 33% (w/v) chloral hydrate at 100°C . The extract did not stain dark blue with iodine and had a more intense absorption maximum at 890cm^{-1} than the original cell wall, indicating preferential extraction of β -glucans.

(c) With aqueous alkali. Cell walls were extracted with 1M-KOH and further fractionated on the basis of water solubility as described in the Materials and Methods section. The properties of the three fractions are given in Table 6. All the fractions contained small amounts of rhamnose, xylose and arabinose and were stained dark blue by iodine solution. Fraction *A* contained 55% of the galactose and 44% of the mannose originally present in the cell wall. The galactomannan components of fractions *A* and *B* could not be precipitated by Fehling's solution or barium hydroxide (Peat, Whelan & Edwards, 1961; Meier, 1961). Glucosamine was found mainly in fraction *C*, but traces were also found in fractions *A* and *B*, when

acid hydrolysates were examined by paper chromatography (solvents *B* and *C*, detecting reagents *a* and *c*). All the fractions were stained dark blue by iodine and had peaks at 850cm^{-1} and 890cm^{-1} ; however, the lower optical rotation of fraction *A* suggests that it contains a higher proportion of β -glucan.

If cell walls were heated with 0.05M-sodium acetate buffer, pH 5.0, for 3 h at 75°C as described by Bacon, Davidson, Jones & Taylor (1966) and then fractionated with 1M-KOH as before, the amounts and compositions of the fractions were very similar to those obtained without this treatment, indicating the absence of a surrounding membrane of the type suggested for yeast cells by Bacon *et al.* (1966). However, if cell walls were first incubated with Pronase, as described above, and then fractionated with 1M-KOH, the amount of the water-soluble fraction increased to 27.5%. This combined treatment resulted in release of 70% of the cell wall galactose as a water-soluble polymeric fraction.

Further fractionation of fractions *A* and *B*, (Scheme 2) enabled the isolation of fractions containing predominantly α - and β -glucose linkages respectively. Fraction *AA* still was stained dark blue by iodine and although it gave a single peak when examined by analytical ultracentrifugation, it could be further fractionated. Addition of 0.1M-iodine gave a precipitate, which was washed with

Table 5. *Properties of fractions obtained by extraction of cell walls with ethylenediamine*

Fraction	Weight (%)	$[\alpha]_D$	Composition (% anhydro sugar)		
			Total hexose	Glc	Gal
<i>EA</i> , Ethylenediamine-soluble, water-soluble	20	-25°	63	50	4.7
<i>EB</i> , Ethylenediamine-soluble, water-insoluble	15	$+120^\circ$	78	70	3.7
<i>EC</i> , Ethylenediamine-insoluble, water-insoluble	60	—	83	75	3.1

Table 6. *Properties of fractions obtained by extraction of cell walls with 1M-potassium hydroxide*

Fraction	Weight (%)	$[\alpha]_D$	Composition (% anhydro sugar)			
			Total hexose	Glc	Gal	Man
<i>A</i> , Alkali-soluble, water-soluble	20	$+15^\circ$	68	46	10.5	6.2
<i>B</i> , Alkali-soluble, water-insoluble	36.5	$+136^\circ$	86	81	1.7	0.8
<i>C</i> , Alkali-insoluble, water-insoluble	36.0	—	77	70	3.1	1.5
Recovery	92.5

Scheme 2. Separation of α - and β -glucan fractions from *F. amygdali* cell walls.

ethanol and freeze-dried to give fraction AA₁ (0.9g), $[\alpha]_D + 40^\circ$, which gave a deep blue colour with iodine, similar to that given by amylose. Its low rotation suggests that it may contain both α - and β -glucose linkages. Addition of 2 vol. of ethanol to the supernatant, after removal of fraction AA₁, caused precipitation of fraction AA₂, (1g), $[\alpha]_D - 71^\circ$, which did not stain with iodine solution; its very low rotation suggested that it was composed of β -linked glucose residues.

Fraction BB, $[\alpha]_D + 236^\circ$, which also stained dark blue with iodine, was composed of glucose and small amounts of galactose, mannose, xylose, arabinose and rhamnose. Examination by ultracentrifugation showed two components with s_{20}^0 values (in 1M-NaOH) of 2S and 6S. The structural

characterization of these two components is described in the following paper (Obaidah & Buck, 1971).

Extracellular polysaccharides. Polysaccharides were isolated from filtrates of the cultures used for cell-wall preparation by precipitation with 2 vol. of ethanol. Three fractions were obtained after dialysis against water. Fraction 1 was diffusible; fraction 2 was non-diffusible and water soluble; fraction 3 was non-diffusible and precipitated on dialysis. Only fraction 2 stained dark blue with iodine. The carbohydrate composition of the fractions is given in Table 7. Although fractions 2 and 3 had a similar composition, their i.r. spectra were markedly different. Fraction 2 had absorption maxima at 810 cm⁻¹ and 920 cm⁻¹ (weak), whereas

Table 7. Carbohydrate composition of extracellular polysaccharide fractions

Fraction	Weight (% of total hexose)					
	Glc	Gal	Man	Ara	Xyl	Rhamnose
1, Diffusible	30	Trace	10	0	50	10
2, Non-diffusible, water-soluble	11	37	26	0	Trace	26
3, Non-diffusible, precipitated on dialysis	11	37	26	0	Trace	26

fraction 3 had maxima at 790cm^{-1} , 810cm^{-1} and 890cm^{-1} .

DISCUSSION

The cell walls of *F. amygdali* consisted of 85% polysaccharides with small amounts of lipid (5%) and protein (4–6%); these components accounted for 94–96% of the cell-wall dry weight.

Seven of the 12 naturally occurring sugars identified as fungal cell-wall constituents (Bartnicki-Garcia, 1968) were found. D-Glucose, D-glucosamine and D-mannose are found in most fungal cell walls (Crook & Johnston, 1962; Bartnicki-Garcia, 1968). D-Galactose is fairly characteristic of the Ascomycetes, whereas L-rhamnose has been reported in the cell walls of *Penicillium chrysogenum* (Hamilton & Knight, 1962) and a number of Phycomycetes (Novaes-Ledieu, Jimenez-Martinez & Villanueva, 1967). There have been very few reports of the occurrence of pentoses in fungal cell walls. Hamilton & Knight (1962) found small amounts of xylose in *P. chrysogenum* cell walls and it was reported in *Polystictus sanguineus* (Crook & Johnston, 1962). Arabinose has been found in cell walls of *A. niger* (Johnston, 1965) and *P. chrysogenum* (Greenawalt, 1960) cited by Troy & Koffler (1969).

The importance of peptide chains in the structure of the cell wall was suggested by the increased total solubility of cell walls in alkali and dimethyl sulphoxide, after incubation with Pronase. Moreover, treatment of cell walls with Pronase, in addition to removing 67% of cell-wall protein, resulted in the release of 30% of the galactose, 11% of the mannose, 17% of the rhamnose and 38% of the P from the cell walls as a heterogeneous, water-soluble, non-diffusible fraction. The detection of sugar phosphates (mainly galactose 6-phosphate) in acid hydrolysates of this fraction suggests that phosphodiester linkages may serve to link together smaller polysaccharide units. Such linkages have been postulated to hold together mannan units in the yeast cell wall (Sentandreu & Northcote, 1968)

and are found in a number of fungal extracellular polysaccharides (Gorin & Spencer, 1968). It is thus possible that the high-molecular-weight fraction, released from cell walls by *H. pomatia* enzymes, which contain a wide range of carbohydrases, but little or no proteases (Holden & Tracey, 1950; Anderson & Millbank, 1966), consisted of relatively small polysaccharide units, linked together both by phosphodiester linkages and peptide chains. This type of structure has been found in other fungal cell walls, e.g. Cawley & Letters (1968) isolated a phosphoglycopeptide after incubating yeast cell walls with Pronase, and Lloyd (1970) has isolated a galactomannan, containing covalently bound phosphate and peptide, from the yeast form of *Cladosporium werneckii*.

Extracellular polysaccharides were isolated from culture filtrates of *F. amygdali*. The dialysis residue had similar proportions of galactose, mannose and rhamnose as the non-diffusible fraction released when cell walls were incubated with *H. pomatia* enzymes. The almost total absence of xylose from these two fractions is probably because of the lower molecular weight and the diffusion properties of the xylose-containing oligosaccharides. In agreement with this the diffusible fraction of the extracellular polysaccharide contained 50% of xylose. It is possible that the extracellular polysaccharides were released by enzyme action on cell walls during growth. It has been suggested that lytic enzymes may occur at the sites of cell-wall formation and act together with the synthetic enzyme systems (Bartnicki-Garcia, 1968).

Most of the cell-wall glucosamine could be accounted for as chitin. However, small amounts of glucosamine were also present in fractions soluble in alkali and soluble in water. Sentandreu & Northcote (1968) obtained evidence suggesting that peptide chains were linked to the high-molecular-weight mannan component of yeast cells by an N-glycosyl bond between N-acetyl-D-glucosamine and asparagine. Further work is necessary to determine if glucosamine has a similar function in *F. amygdali* cell walls.

Glucans were the major components of the cell wall, present in α -(1 $\rightarrow$ 3)-, α -(1 $\rightarrow$ 4)-, β -(1 $\rightarrow$ 3)- and β -(1 $\rightarrow$ 6)-glucopyranose linkages. β -(1 $\rightarrow$ 3)-, β -(1 $\rightarrow$ 6)-Glucans are of very common occurrence in fungal cell walls (Bartnicki-Garcia, 1968) and α -(1 $\rightarrow$ 3)-glucans, although reported comparatively recently as fungal cell-wall constituents, may be fairly widespread (Bacon *et al.* 1968). However, although glycogen-like polymers have been reported in cell walls of *Dictyostelium discoideum* (Ward & Wright, 1965) and the microcyst walls of *Polysphondylium pallidum* (Toama & Raper, 1967), and a glucan, stated without evidence, to contain β -(1 $\rightarrow$ 3) and α -(1 $\rightarrow$ 4) linkages, was isolated from *Aspergillus oryzae* cell walls (Horikoshi & Iida, 1964), the α -(1 $\rightarrow$ 4)-glucan in *F. amygdali* appears to be the first report of linear chains of α -(1 $\rightarrow$ 4)-glucopyranose residues, staining dark blue with iodine, in a fungal cell wall. This glucan appears to be an important structural component, since cell walls appeared much thinner after α -amylase treatment, and it could not be extracted from the cell walls by aqueous chloral hydrate, an excellent solvent for starch (Bourne, Stacey & Wilkinson, 1950; Bourne & Weigel, 1965).

Our results suggest that the *F. amygdali* cell wall consists of a backbone of chitin and α -(1 $\rightarrow$ 3)-glucan, surrounded by a matrix of β -(1 $\rightarrow$ 3)-, β -(1 $\rightarrow$ 6)-glucan, α -(1 $\rightarrow$ 4)-glucan and heteroglycan units, containing galactose, mannose, rhamnose, xylose and arabinose, which may be linked to each other, or to the other polysaccharide components, by glycoside or phosphodiester linkages or by peptide chains. *H. pomatia* digestive enzymes degraded the chitin, β -(1 $\rightarrow$ 3)-, β -(1 $\rightarrow$ 6)-glucan and α -(1 $\rightarrow$ 4)-glucan, and released the soluble heteroglycan fraction, but were without action on the α -(1 $\rightarrow$ 3)-glucan. The resistance of the α -(1 $\rightarrow$ 3)-glucan component to these enzymes may explain the low efficiency of release of protoplasts by this system. It is possible that more efficient protoplast preparation may result from the combined use of *H. pomatia* enzymes and an α -(1 $\rightarrow$ 3)-glucanase, e.g. the *Trichoderma viride* enzyme (Hasegawa, Nordin & Kirkwood, 1969).

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