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Protoplast Formation and Yeast Cell-Wall Structure

THE ACTION OF THE ENZYMES OF THE SNAIL, *HELIX POMATIA*

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1. The digestive juice of the snail *Helix pomatia* was used in the study of the degradation of isolated cell-wall preparations from a strain of *Saccharomyces carlsbergensis*. 2. The crude enzyme system was fractionated by gel filtration and the activities of the more specific fractions thus obtained were examined. 3. Results are discussed with respect to (*a*) the nature of various factors that are essential for protoplast formation and cell-wall dissolution and (*b*) structures envisaged in yeast cell walls that are responsible for the observed variations in susceptibility to attack by snail juice.

Enzyme preparations from several microbial sources have been used in the elucidation of yeast cell-wall composition (e.g. Mendoza & Villanueva, 1962; Bacon, Milne, Taylor & Webley, 1965). One of the richest sources of lytic enzymes for yeast cell wall is the digestive juice from the snail, *Helix pomatia*. Variations in susceptibility to attack by snail juice have been observed between one yeast strain and another of the same species and also between logarithmic-phase and stationary-phase cells of the same strain (Eddy & Williamson, 1957; Millbank & Macrae, 1964); it seems likely that these phenomena result from differences in cell-wall composition.

The present work, where snail juice was used mainly in the study of 'age' variation, is part of a programme of investigation into yeast cell-wall structure and the mechanism of protoplast formation.

Snail digestive juice is known to be a complex mixture of enzymes (Holden & Tracey, 1950) and some fractionation is desirable for the elucidation of the essential factors involved in the action on yeast cell wall. Preliminary work showed that a fractionation into more specific components could be achieved by gel filtration (Millbank & Macrae, 1964). The present paper describes the results of a more effective fractionation than has been achieved hitherto.

MATERIALS AND METHODS

Organisms. Strains of *Saccharomyces carlsbergensis* (N.C.Y.C. 74S) and *Saccharomyces cerevisiae* (N.C.Y.C. 239) were supplied by the British National Collection of Yeast Cultures. They were maintained in liquid culture in the growth medium and subcultured every 4 weeks. After inoculation the stock cultures were incubated at 25° for 72 hr. and kept subsequently at room temperature (20°).

Growth medium. The medium comprised malt extract (0·3%), yeast extract (0·3%), glucose (1·0%) and peptone (0·5%) in distilled water (Wickerham, 1951).

Growth conditions. Roux flasks containing medium (400ml.) were inoculated with 0·5ml. of a mature culture and incubated at 25° on a reciprocating shaker (3cm. amplitude, 80cyc./min.). LP cells† were obtained from cultures after 16hr., when the cell density was approx. 0·4mg. dry wt./ml., by centrifugation at 1000*g* for 3min. SP cells were obtained by harvesting cultures after 42–50hr., when the cell density was approx. 5 mg. dry wt./ml. The cells were washed with buffer and resuspended as appropriate. The citrate–phosphate buffer used throughout was at pH5·8 and refers to that of McIlvaine (1921) diluted 1:5.

Preparation of cell-wall material. Washed yeast cells (30–50mg. dry wt.) suspended in 10% (w/v) sucrose soln. (6ml.) were shaken with Chance Ballotini no. 12 glass beads (approx. 6·8g.) for 10–12min. in a Mickle disintegrator. This treatment gave 95–99% disruption estimated by phase-contrast microscopic observation. The resultant turbid suspension was removed from the beads, which were then washed with further additions of sucrose soln. until the supernatant liquid was clear. The cell suspension, including washings, was then centrifuged at 1000*g* for 5min. to separate the walls from the cell contents. The sediment was washed repeatedly (eight to ten times) with sucrose soln. until microscopic observation showed the cell walls to be free from protoplasmic debris and other unwanted material. Cell-wall material was finally washed with and suspended in distilled water and the dry wt. of the suspension determined by heating samples at 105°. Suspensions were stored at 2°.

Snail digestive juice. This (suc digestif d'*Helix pomatia*) was obtained from Industrie Biologique Française (Genne-

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† Abbreviations: LP cells, early logarithmic-phase cells; SP cells, stationary-phase cells.

villiers, Seine, France) and was found to contain approx. 200mg. dry wt. of material/ml.

Fractionation of snail enzymes. A solution of snail-juice material (100mg.) in 0·85% NaCl soln. (saline) was applied to a column (45cm. × 3·1cm.) containing the gel prepared by swelling Sephadex G-100 (bead form, fine grade; Pharmacia Ltd., London, W. 13) in saline. The column had been previously equilibrated at 4°. Elution was effected with saline at 4° at a flow rate of 35ml./hr.; 3ml. samples of the eluate were collected.

Chromatography. Descending paper chromatograms were prepared at room temperature with Whatman no. 1 paper and either (A) ethyl methyl ketone–acetic acid–water (9:1:1, by vol.; satd. with boric acid) (Rees & Reynolds, 1958) as solvent or (B) ethyl acetate–pyridine–water (10:4:3, by vol.) (Whistler & Hickson, 1955).

Aniline phthalate (Partridge, 1949) (1) or $AgNO_3$–NaOH (Trevelyan, Procter & Harrison, 1950) (2) was used as spray reagent.

Acid hydrolysis. Acid hydrolysis was carried out by heating a 0·5% solution of cell-wall material in 2N-H_2SO_4 at 105° for 3hr. Hydrolysates were neutralized with solid $BaCO_3$ and concentrated by rotary evaporation.

Carbohydrate estimations. (*a*) Total carbohydrate was estimated by the anthrone method of Hall (1956), the method being calibrated with both glucose and mannose as standards; (*b*) glucose was estimated by the method of Fleming & Pegler (1963); (*c*) mannose was estimated by difference.

Protein estimation. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951); bovine plasma albumin was used as standard.

Enzyme assay. Hydrolysis of cellobiose, gentiobiose and laminaribiose was estimated by measuring the glucose content of assay mixtures by the method of Fleming & Pegler (1963). β-Acetylaminodeoxyglucosidase (β-2-acetylamino-2-deoxy-D-glucoside acetylaminodeoxyglucohydrolase, EC 3.2.1.30) activity was estimated by measuring the liberation of *p*-nitrophenol from *p*-nitrophenyl *N*-acetyl-β-glucosaminide (Borooah, Leaback & Walker, 1961; Woollen, Heyworth & Walker, 1961). Phosphomonoesterase and phosphodiesterase were estimated by measuring E_{440} of samples of the assay mixtures after treatment with an equal volume of 0·1N-NaOH. Proteinase was assayed by the method of Anson (1938).

RESULTS

Analysis of cell-wall preparations

(*a*) Carbohydrate. Total carbohydrate, estimated in acid hydrolysates of a number of preparations of either LP or SP cell-wall material of *S. carlsbergensis*, was found to be approx. 85% of the initial weight of the wall. In LP material the glucose/mannose ratio was consistently about 1:1·2; the ratio in SP material was 1:1·7.

(*b*) Protein. Both LP and SP materials gave values of approx. 10–12% for total protein.

Experiments with crude enzyme

Cell-wall material (2mg./ml.) or whole cells (5mg./ml.) together with crude enzyme (5mg./ml.) in citrate–phosphate buffer were incubated at 30° for 90min. 0·6M-Mannitol was used as osmotic stabilizer for whole cells.

Effect on cells and cell walls of S. carlsbergensis. This strain is known to form protoplasts readily under the appropriate conditions (Eddy & Williamson, 1957; Millbank, 1963).

(*a*) Whole cells. Microscopic observation showed that with LP cells complete lysis had occurred, i.e. 100% conversion into protoplasts. No protoplast formation was observed with SP cells.

(*b*) Cell walls. With LP cell walls the bulk of the material went into solution. After removing the small amount of residue by centrifugation, the supernatant liquid and acid hydrolysates of both the supernatant and the residue were subjected to paper-chromatographic analysis (solvents A and B, spray reagents 1 and 2 respectively). Whereas free glucose but no mannose was detected in the supernatant liquid, after hydrolysis both sugars were found in approximately equal amounts; only traces of glucose were observed in the hydrolysate of the residue. In a parallel experiment with cell-wall material from SP cells, only glucose was observed in the supernatant liquid. Glucose and a smaller amount of mannose were detected in the hydrolysed supernatant and glucose plus a greater amount of mannose in the hydrolysed residue.

Effect on cells and cell walls of S. cerevisiae. This strain does not form protoplasts with snail enzyme under the given conditions (Millbank & Macrae, 1964).

(*a*) Whole cells. Microscopic observation revealed little or no attack by snail enzymes; no protoplasts were formed. Chromatographic analysis of the supernatant liquid and its hydrolysate showed that only glucose was released. In these trials mannitol was omitted.

(*b*) Cell walls. Material derived from LP cells of this strain gave results similar to those obtained above with cell wall prepared from SP cells of *S. carlsbergensis*.

Experiments with fractionated enzymes

Snail digestive juice was fractionated on a column of Sephadex G-100. Fig. 1 shows the E_{280} values of the samples eluted from the column (Unicam SP.500 spectrophotometer, 0·5cm.cuvette, saline blank); the first 30ml. was discarded. Identical elution patterns were obtained from numerous runs.

Effect on cells and cell walls of S. carlsbergensis. The digests were similar to those above with 1·5ml. samples of the individual eluates replacing the crude enzyme. Incubation time and temperature were as before.

(*a*) Whole cells. Microscopic observation showed

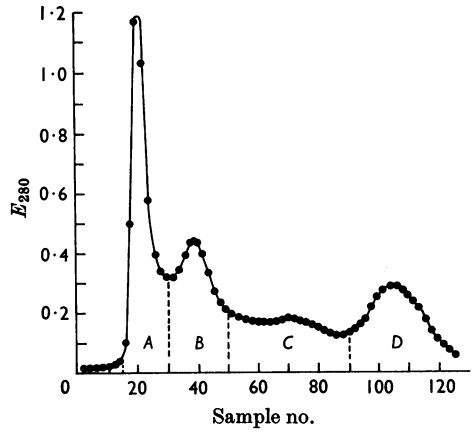

Fig. 1. Gel filtration of snail juice (100mg.) on a Sephadex G-100 column (45cm. × 3·1cm.) in saline. Samples (3ml.) were collected and E_{280} values of the even-numbered samples are recorded.

that protoplasts were only formed from LP cells with samples 60–80. Samples 50–60 were combined, as were samples 80–90. Whereas neither of the solutions thus obtained was capable of producing protoplasts when acting on its own, when they were mixed together in equal proportions 1·5ml. samples of the mixture effected protoplast formation under the given conditions.

(*b*) Cell walls (LP). After incubation any undissolved material was removed by centrifugation and the supernatant liquid was desalted by treatment with ion-exchange resins. The samples (15–30) comprising the initial peak (fraction *A*) produced limited attack on the cell-wall material. Chromatographic analysis (solvent A, spray reagent 1) showed glucose but no mannose in either the supernatant liquid or its hydrolysate. Samples 30–50 (fraction *B*) again exhibited limited attack; only glucose was detected with the earlier samples, whereas the later samples of this group yielded free glucose in the supernatant liquid and glucose plus a smaller amount of mannose in its hydrolysate. Samples 50–90 (fraction *C*) produced almost complete solubilization of the cell-wall material, in particular samples 60–80. With these fractions two further components (R_{Glc} 0·28 and 0·14 in solvent A; mannose has R_{Glc} 1·40) were detected in the supernatant liquid. The identities of these substances are considered below. Acid hydrolysates of the supernatant liquid contained glucose and mannose in approximately equal amounts. The remainder of the samples (fraction *D*) exhibited very limited activity.

Enzymic activities of the samples. The activities examined were selected on the basis of their being possible participants in the degradation of structures known or thought likely to be present in yeast cell wall. All assay mixtures in citrate–phosphate buffer in a total volume of 1·0ml. were incubated at 30°; activities were estimated as given in the Materials and Methods section.

Cellobiase, gentiobiase and laminaribiase. Assay mixtures containing 0·2ml. of the individual samples from the Sephadex column were incubated for 15min. together with 200μg. of the appropriate disaccharide. Hydrolysis of all three substrates occurred only with samples 10–40 with maximum activity in samples 20–30.

β-Acetylaminodeoxyglucosidase. Column samples (0·2ml.) together with 1mg. of *p*-nitrophenyl *N*-acetyl-β-glucosaminide were incubated for 1hr. Hydrolysis occurred with samples 15–35; there was a sharp peak in activity with sample 21.

Phosphomonoesterase and phosphodiesterase. The digests consisted of 0·5ml. of the column samples plus 2·5mg. of either disodium *p*-nitrophenyl phosphate or sodium bis-(*p*-nitrophenyl) phosphate and were incubated for 30min. Enzymic activity towards either substrate was confined to samples 23–45 with maximum activity in sample 33.

No proteolytic activity was detected in any of the samples.

A number of replicate fractionations was carried out and by reference to the E_{280} distribution pattern (cf. Fig. 1) the corresponding samples were combined. The combined samples were then further grouped into four fractions *A–D* corresponding to the fractions described above. These materials were concentrated by freeze-drying, desalted by passage of the concentrated solutions through columns containing ion-retardation resin AG-11A8 (Bio-Rad Laboratories, Richmond, Calif., U.S.A.) and the desalted solutions freeze-dried. Examination of the u.v. spectra of aqueous solutions of these material showed that, whereas fractions *A*, *B* and *C* exhibited an absorption maximum at 280mμ, fraction *D* showed no specific absorption at this wavelength.

Activity of the freeze-dried fractions. (*a*) Cells and cell walls of *S. carlsbergensis* (LP). The action of the freeze-dried fractions on cells and cell walls was found to be similar to that described above for the individual samples comprising the corresponding groups, i.e. protoplasts were only formed from whole cells under the action of fraction *C* and, whereas this fraction brought about almost complete dissolution of isolated cell walls, fractions *A* and *B* showed only limited attack. Fraction *D* was virtually inactive and was not examined further.

(*b*) Model substrates. The assay procedures were as given above with freeze-dried preparations (0·5mg./ml.) in place of the individual samples of

the column eluate. The activities were found to be as expected, i.e. hydrolysis of cellobiose, gentiobiose, laminaribiose and *p*-nitrophenyl *N*-acetyl-β-glucosaminide occurred mainly with fraction *A* with less activity in fraction *B* and no activity in fraction *C*. The phosphatases were mainly located in fraction *B* with slight activity in fraction *A* and no activity in fraction *C*; similar results were obtained for lipase activity as measured with Tween 20 as substrate (Bier, 1955).

(*c*) Degradation of carbohydrate components of cell wall. Cell-wall materials (2mg./ml.) derived from either LP or SP cells of *S. carlsbergensis* were incubated with each of the freeze-dried preparations *A*, *B* and *C* (1mg./ml.) in citrate–phosphate buffer at 30° for 90min.; the supernatant liquids and residues were analysed chromatographically for carbohydrate constituents. Fraction *A* liberated only glucose from either cell-wall preparation, leaving the bulk of the glucan and the mannan undissolved. The action of fraction *B* was similar to that of fraction *A* except that a small amount of mannan was liberated, i.e. mannose was detected in acid hydrolysates of the supernatant liquid. With fraction *C*, however, there was virtually complete dissolution of LP cell-wall material. Here, in addition to glucose and mannan, other reducing sugars were found in the supernatant liquid including those with R_{Glc} 0·28 and 0·14 in solvent A and R_{Glc} 0·74 and 0·42 in solvent B. Acid hydrolysates of these components showed only glucose. Authentic samples of gentiobiose and laminaribiose had R_{Glc} 0·14 and 0·28 respectively in solvent A and R_{Glc} 0·42 and 0·74 respectively in solvent B. On more prolonged incubation (3–4hr.) of the cell-wall material with fraction *C*, further components of the supernatant liquid with R_{Glc} 1·30 and 0·52 in solvent B were detected; the latter was not characterized. The component with R_{Glc} 1·30, which was slow to react with spray reagent 2, gave a purple–violet colour with the Elson–Morgan reagents (Partridge, 1948) and its chromatographic mobility was comparable with that of *N*-acetylglucosamine. Only glucose was detected in acid hydrolysates of the small amount of residue after attack on LP cell-wall material by fraction *C*. The products of attack by fraction *C* on SP cell-wall material were similar to those from LP material. In this case there was considerable residue found to be rich in mannan.

(*d*) Cell-wall protein. The residues after incubation of the cell-wall preparations with the various freeze-dried fractions were washed thoroughly to remove any contaminating material and the protein contents examined. Whereas with fractions *A* and *B* little or no protein was solubilized from either cell-wall preparation, i.e. the residues contained the bulk of the original protein present in the untreated walls, with fraction *C* virtually all the protein went into solution from LP cell-wall material. However, about 80% of the protein originally present in SP cell wall remained undissolved by fraction *C*.

(*e*) Action of the freeze-dried fractions on soluble laminarin. Assay mixtures containing laminarin (0·5mg./ml.) and each of the snail-juice fractions individually (0·5mg./ml.) in citrate–phosphate buffer were incubated at 30°. Samples were withdrawn at intervals, the reaction was stopped by heating and the glucose content of samples was determined by the method of Fleming & Pegler (1963). The glucose content of the substrate was measured in acid hydrolysates. Whereas fractions *A* and *B* gave almost complete conversion into glucose in 3 and 2hr. respectively, fraction *C* liberated only about 80% of the available glucose in 3hr. and this value did not increase on prolonged incubation. The products of the action of snail-juice fractions on laminarin were studied. Mixtures of substrate (8mg./ml.) and enzyme (1mg./ml.) in citrate–phosphate buffer were incubated at 30°. Samples were withdrawn at intervals, the reaction was stopped by heat treatment and samples subjected to paper-chromatographic analysis with solvent B and spray reagent 2. It was found that throughout the course of the reactions glucose was the only detectable product under the action of either fraction *A* or *B*. Fraction *C*, on the other hand, gave rise to glucose, laminaribiose, laminaritriose (the chromatographic mobilities of which were comparable with authentic samples) and higher oligosaccharides in the early stages of the reaction (up to 3hr. incubation) and yielded glucose and laminaribiose as the main products on prolonged incubation (18hr.).

(*f*) Action of the freeze-dried fractions on the polysaccharide luteose. A series of experiments was carried out with luteose (kindly supplied by Professor D. J. Manners) as substrate; the assay mixtures and conditions were similar to those used above with soluble laminarin. Fractions *A*, *B* and *C* acting individually released glucose more slowly than from laminarin; thus the amount was about 6, 6 and 15% of the theoretical maximum respectively after 1hr. and approx. 75, 75 and 40% respectively on prolonged incubation (18hr.). However, a combination of all three fractions (each 0·5mg./ml.) eventually produced almost complete conversion into glucose (53% after 1hr. incubation). Again with fractions *A* and *B* glucose was the only detectable end product throughout the course of the reactions. With fraction *C*, in addition to glucose, gentiobiose (the chromatographic mobility of which was comparable with that of an authentic sample) and other reducing components (R_{Glc} 0·23 and 0·10 in solvent B) were detected in the early

stages of the reaction and were present on prolonged incubation (18 hr.).

Effect of 2-mercaptoethanol. Samples of a suspension of SP cell-wall material from *S. carlsbergensis* (5 mg./ml.) in tris–hydrochloric acid buffer, pH 7·5 (0·02 M), with or without 2-mercaptoethanol (0·06 M), were incubated at 30° for 1 hr. The wall material was then recovered by centrifugation, washed twice on the centrifuge with citrate–phosphate buffer and samples (2 mg./ml.) were treated with snail-juice fraction *C* (1 mg./ml.) in citrate–phosphate buffer for 90 min. at 30°. Whereas the suspensions of cell wall that had not been pretreated with mercaptoethanol remained turbid, those of the treated material became greatly clarified under the action of fraction *C*. Microscopic observation revealed that the former contained material with the general shape of the original cell wall; no such material was observed in the treated samples. In a parallel experiment intact SP cells (5 mg./ml.) were similarly treated with 2-mercaptoethanol in tris–hydrochloric acid buffer. On subsequent incubation with snail-juice fraction *C* in citrate–phosphate buffer plus mannitol the treated cells readily formed protoplasts, whereas SP cells preincubated with tris–hydrochloric acid buffer in the absence of mercaptoethanol did not form protoplasts on subsequent treatment with fraction *C*.

DISCUSSION

The action of snail juice on cell-wall material derived from yeast cells in a state where protoplasts can be readily formed results in virtually complete degradation of the cell-wall glucan with consequent dissolution of the mannan. The mannan does not appear to be extensively degraded. Millbank & Macrae (1964) reported that under similar conditions mannose occurred among the reaction products. However, it has since been shown (F. B. Anderson & J. W. Millbank, unpublished work) that the free hexose arises from degradation of mannan as a result of the deproteinization procedure employed in the earlier work. That mannan is not extensively degraded is in agreement with the findings of Eddy (1958*a*). With cell-wall material from yeast in a state where protoplasts are not formed under the action of snail juice (SP cells of *S. carlsbergensis*; LP cells of *S. cerevisiae*) the glucan is again degraded. In this case, however, a residue rich in mannan remains undissolved. These results indicate differences in the nature of the mannan between the two types of cell wall; analysis of the wall materials also shows a higher mannan content for the resistant cell wall.

Whole snail juice subjected to gel filtration yields three major fractions characterized by their action on yeast cells, cell-wall materials and model substrates. Only one of these (fraction *C*) has the ability to form protoplasts from LP cells of *S. carlsbergensis* and to bring about extensive degradation of cell-wall material derived from such cells. As this fraction is devoid of the lipase and phosphatases, present in the original snail juice and located in fractions *A* and *B*, and of proteinase, it seems that such enzymes do not play an essential role in cell-wall degradation.

The products of the action of fraction *C* on cell-wall materials differ from those of the whole snail juice in that the glucan is incompletely hydrolysed to glucose. The two other fractions *A* and *B* contain the activities necessary for the hydrolysis of the β-linked glucose disaccharides remaining after attack by fraction *C*. Fractions *A* and *B* exhibit only limited attack on the cell-wall glucan, glucose being the sole detectable product. The results are explicable if fractions *A* and *B* attack cell-wall glucan by exo-glucosidase action, such action being curtailed by the presence of branch points in the molecule. Fraction *C*, on the other hand, may act in a random fashion and in this way by-pass such branch points; it may also hydrolyse any branch-point linkages. Support for this hypothesis has been gained from the study of the action of the snail-juice fractions on the β-(1→3)- and β-(1→6)-linked glucose polysaccharides laminarin and luteose (Anderson, Haworth, Raistrick & Stacey, 1939) respectively. Whereas fractions *A* and *B* degrade both polysaccharides extensively in a stepwise manner with the liberation of glucose alone, fraction *C* acts in a random fashion. The approximately complete conversion of luteose, which is known to be a branched structure, into glucose by the combined action of the three fractions may be indicative of some de-branching activity being present in fraction *C*. Both linear and branched structures have been suggested for yeast cell-wall glucan (for a review, see Phaff, 1963); however, the present postulation of a branched structure is supported by the recent findings of Manners & Patterson (1966). The endo-β-(1→3)- and endo-β-(1→6)-glucanase present in fraction *C* may be analogous to those isolated by Tanaka & Phaff (1965) from the yeast cell-wall lytic enzymes produced by a strain of *Bacillus circulans*. A separation of the snail-juice activities is being undertaken to determine whether both are essential for cell-wall degradation and protoplast formation. It was observed that, whereas neither the initial nor the last eluate samples included in fraction *C* were capable of protoplast formation on their own, they were active in this respect when combined. This indicates that at least two enzymic activities are essential for protoplast formation. Such activities may well be endo-β-(1→3)- and endo-β-(1→6)-glucanase and

perhaps a partial separation has been achieved on the Sephadex column.

The release of *N*-acetylglucosamine on treatment of cell wall with snail-juice fraction *C* is consistent with the findings of Eddy (1958*a*). The role of the amino sugars in yeast cell-wall structure is as yet unestablished. Various references to the possible presence of chitin in yeast cell wall are to be found in the literature (for reviews, see Eddy 1958*b*; Phaff, 1963). It has also been suggested that the amino sugar may provide the link between protein and carbohydrate in the walls (Eddy, 1958*a*; Korn & Northcote, 1960).

The fact that fraction *C* dissolves all the protein in LP cell walls but only 20% in SP cell walls suggests either that the protein itself differs in the two types of cell wall, or that it is differently linked. It has been shown by Davies & Elvin (1964) that protoplast formation from *Saccharomyces fragilis* under the action of snail juice is enhanced by pre-incubation of the cells with 2-mercaptoethanol. The present work shows that pretreatment of SP cells of *S. carlsbergensis* and cell-wall materials derived from such cells with 2-mercaptoethanol results in protoplast formation and cell-wall dissolution respectively on subsequent incubation with snail-juice fraction *C*. From this it seems that the thiol has in some way modified the mannan and perhaps the protein of the SP cell-wall, rendering it as susceptible to attack by the snail-juice enzymes as is the LP cell wall. Bacon *et al.* (1965) from their studies on the effect of 2-mercaptoethanol on the lytic enzymes from *Cytophaga johnsonii* postulate that two structural systems exist, either of which when intact preserve the integrity of the yeast cell wall. One of these structures is the glucan, the other composed of mannan–protein complexes associated through disulphide linkages as suggested by Nickerson & Falcone (1956). Extending this hypothesis to the present findings, perhaps the difference between cell wall from either LP or SP cells of *S. carlsbergensis* and perhaps between all yeasts that are either susceptible or non-susceptible to protoplast formation under the action of snail juice lies in the nature of the second of the two proposed structural systems. It is possible that in the susceptible yeasts the disulphide linkages are incompletely formed and degradation of the glucan is sufficient to effect solubilization of the complete cell wall. On the other hand, in the resistant cell wall it is only after the disulphide linkages have been disrupted by such agents as 2-mercaptoethanol that the glucanases can bring about complete dissolution.

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