

The isolation from *Saccharomyces cerevisiae* of two antibacterial cationic proteins that inhibit malolactic bacteria

by

K. J. DICK¹), P. C. MOLAN¹) and R. ESCHENBRUCH²)

¹) Department of Biological Sciences, University of Waikato, Hamilton, New Zealand

²) D.S.I.R. Wine Research Laboratory, Te Kauwhata, New Zealand
Present address: Rongopai Wines, Box 35, Te Kauwhata, New Zealand

Die Isolierung von zwei bakteriziden, kationischen Proteinen aus *Saccharomyces cerevisiae*, die Bakterien des Säureabbaus hemmen

Zusammenfassung: Substanzen mit bakterizider Wirkung wurden sowohl aus dem Kultursubstrat als auch aus angesäuertem Zellextrakt von *Saccharomyces cerevisiae* R107 mit Hilfe von Kationenaustauscher isoliert. Die mit NaOH eluierte Fraktion enthielt zwei unterschiedliche bakterizide Substanzen, eine mit Lysozymeigenschaften, die andere, ein kleines Protein, mit ähnlich hohem isoelektrischen Punkt.

Key words: *Saccharomyces cerevisiae*, yeast, cationic proteins, antibacterial proteins, bacteria, lactic acid, malo-lactic fermentation, growth inhibition.

Introduction

Wine-makers and brewers generally seek to prevent bacterial growth during yeast fermentation because of the resulting spoilage of wine and beer. However, wine-makers seek in some instances to encourage the growth of genera such as *Leuconostoc* and *Lactobacillus* to convert malic acid to lactic acid and thus reduce the acidity of the wine. This malolactic fermentation is regarded as essential for certain types of wine, but at times can be very difficult to get started. One possible explanation for this difficulty is that the wine may be lacking essential nutrient factors for the lactic acid bacteria. Another possible explanation is that inhibitory substances are accumulated in wine. It has been suspected that the reason why malolactic fermentation cannot be started sometimes is because antibacterial factors are produced by the yeast (FORNACHON 1968; LAFON-LAFOURCADE 1973; KING and BEELMAN 1986; LEMARRESQUIER 1987). WIBOWO *et al.* (1988) found that wines produced with some strains of *Saccharomyces cerevisiae* inhibited subsequent growth of *Leuconostoc oenos*, but there were no differences in the levels of ethanol or sulphur dioxide, to which the antibacterial activity had been attributed in the past. Thus some other antibacterial factor produced by particular strains of *Saccharomyces* may be involved in the failure to establish malolactic fermentation.

A yeast with antibacterial properties has been sought in an attempt to inhibit bacterial growth in brewing (BILINSKI *et al.* 1985). Although over 400 strains belonging to 31 yeast genera were screened, only two were found to be antibacterial, and these, non-*Saccharomyces* strains, apparently produced this activity by the modification of methylene blue added to the medium.

Yeast killer factors are well known antimicrobial substances produced by *Saccharomyces* (WOODS and BEVAN 1968) and other genera (PHILLISKIRK and YOUNG 1975). However, these factors are active only against yeasts.

The production by yeasts of substances active against bacteria is less known. Large numbers of strains have been screened for antibacterial activity. One study of 500 found no antibiotic substances (IVANTSKAYA 1959). Another, comprising 153 strains, found only *Candida pulcherrima* to be active, this yeast secreting pulcherriminic acid (MACWILLIAM 1959). The antibacterial factor produced by *Candida guilliermondii* was found in another study to be a cationic substance (BUDAK *et al.* 1982).

Various workers have found antibacterial activity in *S. cerevisiae* (UROMA and VIRTANEN 1947; KOCH 1952; PARFENTJEV 1953, 1958; SKORODUMOVA 1954; MOTZEL and COOK 1958; ROBINSON *et al.* 1958, 1962; SAKAGAMI 1960). The factor responsible for the activity was not characterized in all cases but in one it was reported that it was due to a mixture of unsaturated fatty acids (UROMA and VIRTANEN 1947). Others identified a protein with an isoelectric point of pH 2 (PARFENTJEV 1958) and two apparently cyclic peptides (MOTZEL and COOK 1958; ROBINSON *et al.* 1962).

We screened the wine-making strains of *S. cerevisiae* held in the Ruakura collection to identify any with the potential to inhibit malolactic fermentation. Several strains (R92, R93, R102 and R107) were found to produce antibacterial activity, strain R107 showing the highest activity (MOLAN and ESCHENBRUCH, unpublished work). Because of the variability in the nature of the antibacterial substances from *S. cerevisiae* reported, we decided to isolate and characterize the antibacterial factor from *S. cerevisiae* R107.

Materials and methods

1. Culture and processing of yeast cells

S. cerevisiae R107 was grown for 6 d at room temperature in a sterile complete mineral medium, as described by TOKUYAMA *et al.* (1973), but with the content of glucose increased 10-fold, of biotin 100-fold, minor elements 1000-fold and calcium pantothenate 2-fold. In addition, folic acid (400 mg/l) and vitamin B₁₂ (100 µg/l) were included. Batches of 20 l were grown in two 10-l bottles. Inocula were from fresh cultures maintained by sub-culturing. The bottles were swirled occasionally to suspend the yeast.

On the 7th day after inoculation the yeast cells were separated from the growth medium by continuous flow centrifugation at 30 900 *g* at 4 °C. They were suspended as a thick slurry in distilled water and disintegrated by passing four times through a French pressure cell (Aminco, Silver Springs, MD, U.S.A.) at a pressure of 40–60 mPa. Two volumes of 0.25 M sulphuric acid were added and the suspension allowed to stand for 3 d at room temperature.

2. Assessment of antibacterial activity

Antibacterial activity was assayed by an agar well diffusion assay, using as the test organisms *Escherichia coli* B, *Staphylococcus aureus* and *Bacillus* sp. (similar to *B. subtilis*). The bacterial cultures were each grown for 24 h in 8 ml nutrient broth (Becton Dickson and Co., Fort Worth, Texas, U.S.A.). Each was added to 400 ml nutrient broth

containing 1 % agarose at 45 °C, then the plates were poured immediately. Wells of 5 mm diameter were cut into the plates. These were filled with test solutions and the plates were incubated at 37 °C for 18 h. The antibacterial activity was recorded as the radial extent of the annular clear zone around the well.

A similar method was used to test for activity against lactic acid bacteria, but a different medium was used. Into each petri dish was pipetted 2—5 ml of a 3 to 5-d-old culture of the species in M.R.S. medium (DE MAN *et al.* 1960). This was mixed with 20 ml of M.R.S. medium containing 1.5 % agarose (at 45 °C).

Lytic activity was measured by the method of GOSNELL *et al.* (1975), using 0.02 % (w/v) lyophilized *Micrococcus lysodeikticus* cells (Sigma Chemical Co., St. Louis, MO, U.S.A.) in 0.1 M phosphate buffer, pH 7.0, plus 0.1 % NaCl and 1 % agarose. The mixture was boiled with the bacterial cells present when dissolving the agarose prior to pouring the plates. Wells of 5 mm diameter were cut in the plates. The activity was recorded as the radial extent of the annular zone of clearing around the well.

3. Extraction of antibacterial activity

The antibacterial activity was obtained from the extracellular medium and from the cell extract by passing each in turn through a cation exchange column at pH 3; the activity was bound by the ion exchanger and was subsequently eluted as described below:

(i) From cell extract

The suspension of acidified disintegrated cells was centrifuged at 39 100 *g* for 40 min at 4 °C. The supernatant (about 700 ml) was adjusted to pH 3.0 with NaOH, then passed through a 1.6 × 20 cm column of SP Sephadex C25 (Pharmacia, Uppsala, Sweden) at 4 °C equilibrated with 0.1 M sodium citrate buffer pH 3.0. The column was washed with 100 ml 0.1 M sodium phosphate buffer, pH 7.0. Finally, the bound material was eluted by passing 0.1 M NaOH through the column. Fractions of 5 ml were collected and neutralized with HCl immediately. The fractions were tested for lysozyme activity and activity against the *Bacillus* sp. Also tested for antibacterial activity (after adjustment to pH 7.0 with NaOH) were samples of the material that came straight through the ion exchange column, the material eluted by the wash with phosphate at pH 7.0 and the supernatant and re-suspended pellet of the disintegrated cells.

(ii) From extracellular medium

The 20 l of extracellular medium was subjected to the same ion exchange procedure as the cell extract, except that 200 ml of 0.1 M phosphate buffer, pH 6.0, was used to wash the column after loading and fractions of 10 ml were collected. All fractions were neutralized and tested for lysozyme activity and activity against the *Bacillus* sp.

4. Isolation of antibacterial activity

(i) Gel filtration chromatography

The fractions with antibacterial activity eluted from ion exchange chromatography of the yeast cell extract were pooled and 25 ml was taken and concentrated to 5 ml on a rotary evaporator at 37 °C. The concentrate was adjusted to pH 1.7 with HCl and then chromatographed on a 2.6 × 30 cm column of Sephadex G50 (Pharmacia) eluted with 0.02 M HCl (pH 1.7). Fractions of 5 ml were collected. All fractions corresponding with the peaks obtained were individually neutralized with NaOH and tested for antibacterial and lysozyme activity. In order to detect lower levels of antibacterial activity the

fractions comprising each peak were pooled and concentrated to 5 ml on a rotary evaporator at 37 °C. The concentrates were tested for lysozyme and antibacterial activity.

(ii) Electrophoresis

The fractions with antibacterial activity from ion exchange chromatography were pooled, desalted and concentrated 5-fold by ultrafiltration with a filter of nominal retentivity 500 Da (Diaflow UM05, Amicon, Danvers, MA, U.S.A.). The concentrate was subjected to discontinuous cationic electrophoresis by the method of REISFELD *et al.* (1962), 500 ml being run on a slab of polyacrylamide gel of dimensions 80 × 80 × 3 mm. The sample was applied as a slurry soaked into Sephadex G100 (Pharmacia) in a trough cut in the large-pore gel. A standard of egg-white lysozyme (Sigma Chemical Co.) was run alongside for comparison. Methyl green was used as a tracking dye. After electrophoresis the slab was cut into strips longitudinally and one of the strips and the strip containing the standard were fixed and stained by the method of FAIRBANKS *et al.* (1971). One of the other strips was taken and placed in a petri dish and over it was poured cooled molten agarose gel containing *M. lysodeikticus* cells, prepared as described above for the 'lyso-plate' assay of GOSNELL *et al.* (1975). The gel was incubated at 37 °C overnight.

The concentrate prepared for electrophoresis was also subjected to SDS electrophoresis by the discontinuous method of LAEMMLI (1970).

(iii) Isoelectric focussing

Fractions with antibacterial activity prepared for electrophoresis were used also for isoelectric focussing.

Analytical isoelectric focussing was carried out according to the manufacturer's instructions on Ampholine PAG plates (LKB Chemical Ltd., Bromma, Sweden) with a pH range of 3.5–9.5 extended by placing a 10-mm-wide strip of filter paper soaked in undiluted Ampholine pH 9–11 across the gel 10 mm from the cathodal electrode strip. (This strip was removed after 30 min of focussing.)

Preparative isoelectric focussing in Sephadex 1EF using equipment supplied for this procedure by Pharmacia was carried out according to the manufacturer's instructions. The gel slurry contained 1.5 g Sephadex 1EF, 22.5 ml water, 1.08 ml Ampholine pH 9–11 (LKB Chemicals Ltd.) and 0.12 ml Servalyte pH 4–9 (Serva Feinbiochemica, Heidelberg, Germany) and was spread to a thickness of 1.5 mm on a glass plate 80 × 140 mm. The gel was pre-focussed for 30 min at 4 W, then 300 µl of sample was applied across the centre. After focussing for 3 h at 4 W the proteins were located by inserting the edge of a strip of filter paper into the gel along its length for 1 min, then drying, fixing and staining the paper strip in the same way as the PAG plates. The gel across the width of the plate in the position corresponding to staining on the paper strip was scooped out, neutralized and the protein eluted with water. The eluent was concentrated by rotary evaporation assayed for antibacterial activity.

Immediately after each isoelectric focussing run the pH gradient in the gel was measured with a surface electrode.

5. Characterization of the antibacterial components

(i) Susceptibility to proteolysis

The protease solutions used were chymotrypsin (Type II, from bovine pancreas: Sigma Chemical Co.), 5 mg/ml in 0.1 M phosphate buffer pH 8.0, and actinidin (crystallized from *Actinida sinensis*, kindly donated by Dr. C. J. MOORE, Massey University, New Zealand), 5 mg/ml in 0.1 M phosphate buffer, pH 6.0, with 5 mg/ml dithiothreitol added.

Two samples were subjected to proteolysis. One was a sample from ion exchange chromatography of extracellular medium, which was assayed for lysozyme after proteolysis: (This sample showed no activity against *E. coli* and *S. aureus*.) The other was a sample from ion exchange chromatography of cell extract which was assayed against *E. coli* after proteolysis. (This sample showed no lysozyme activity.) Both samples were concentrated and desalted as described above.

One part of protease solution was added to five parts of sample and the mixtures were placed in the wells of the agar assay plates. Controls included were sample plus protease solution boiled for 3 min and cooled.

(ii) Heat stability

Samples as prepared for proteolysis were also subjected to heating to 35, 45, 55, 65, 80 and 100 °C for periods of 5, 10, 20, 40 and 60 min before cooling and assaying activity. All samples were at pH 6.0 before heating.

(iii) Optimum pH for activity

Samples as prepared for proteolysis were also used to assess the antibacterial activity at various pH values. Plates prepared for lysozyme assay were soaked, at 4 °C for 24 h, in three changes of a 0.3 M phosphate buffer at a pH ranging from 3.0 to 11.0. Plates seeded with *E. coli* were prepared fresh with 0.01 M phosphate buffer and the nutrient broth adjusted to pH values from 4.0 to 10.5. The agarose was added as a sterile solution in water after autoclaving the buffered broth, to avoid hydrolysis of the agarose.

The pH of the plates was measured, at room temperature, with a surface electrode before and after incubation in the assays of the antibacterial activity of the samples.

Results

1. Extraction of antibacterial activity

Prior to ion exchange chromatography the cell extract typically gave a clear zone of 2.5 mm on the *Bacillus* sp. plates and in the lysozyme assay showed low or sometimes no activity. No activity was detected in the extracellular medium or in the re-suspended pellet of disintegrated cells.

No activity was detected in the material that passed straight through the ion exchange column or in the material washed off the column with the phosphate buffer, when chromatographing cell extract or extracellular medium. Elution of the ion exchange column with NaOH yielded antibacterial activity from both. From the cell extract activity was typically eluted over 13 fractions, with a clear zone of 4 mm on the *Bacillus* sp. plates over the central 6 fractions, and with lysozyme activity in fractions 2—8 with clearings of 2 mm in the central 3 fractions. From the extracellular medium activity on the *Bacillus* sp. plates was in fraction 2—9 with clear zones up to 1 mm, this sometimes not being present. However, lysozyme activity was always present, typically eluted over 20 fractions with clearings of 1 mm rising to 1.5—2 mm in the central 9 fractions.

2. Isolation of antibacterial activity

(i) Gel filtration chromatography

A typical elution profile from gel filtration chromatography of concentrated cell extract is shown in the figure. Activity against *Bacillus* sp. (1—4 mm, depending on the

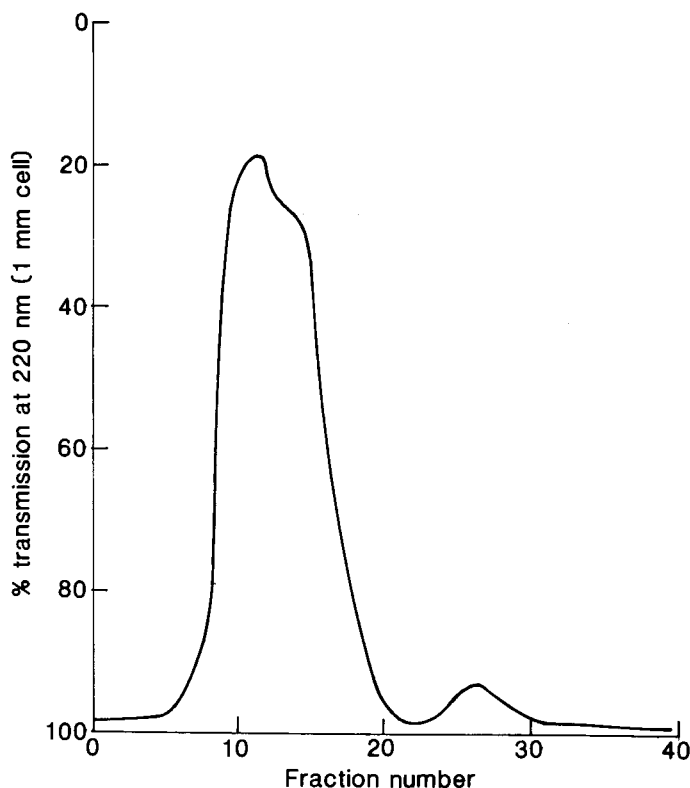
batch of cell extract) was found in the first peak (in fractions 10—15). Batches of cell extract with higher activity gave activity (1—2 mm) in the second peak (in fractions 16—20) also. Lysozyme activity (0.5—1 mm) was found in fractions 13—15 if present at all. After concentrating the fractions lysozyme activity was always detected, in the first peak as well as the second peak. Also, activity against *Bacillus* sp. was found in the third peak after concentration.

(ii) Electrophoresis

Cationic electrophoresis showed the active fractions from ion exchange chromatography to contain several bands of low mobility, a single band of high mobility and a diffuse band with the same mobility as egg-white lysozyme. Characteristically a large amount of tailing of protein was seen in the gel where the yeast proteins had been run.

A clearing of the *Micrococcus* cells in the agarose layer poured over unstained strips of the polyacrylamide gel was seen most times, the region of clearing being found only in the position corresponding to the diffuse band of mobility similar to lysozyme. Sometimes the clearing appeared to be a double band.

SDS electrophoresis of the same material showed the presence of only three bands, two major bands of low molecular weight (moving close to the dye front) and a



Gel filtration chromatography on Sephadex G50 of the antibacterial fraction of yeast cell extract obtained from ion exchange chromatography.

Gelfiltration der bakteriziden Fraktion des Hefezelleextraktes mit Sephadex G50.

band with the same mobility as the egg-white lysozyme standard.

(iii) Isoelectric focussing

Analytical isoelectric focussing of the active fractions from the cell extract showed two closely-spaced major bands at a pH of 11.5 in the gradient. The egg-white lysozyme standard was also found to focus at the same point. Some cell extract preparations contained additional minor bands of lower pI and occasionally an anionic component.

Active fractions from extracellular medium prepared in the same way as the cell extract for isoelectric focussing gave a single band at pH 11.5.

Preparative isoelectric focussing of the cell extract preparation showed a single broader band on the stained paper strip. This band and the band from egg-white lysozyme were at a position corresponding to a pH of 10.2 in the gradient.

The material eluted from the gel scooped from this region was found to have strong activity against *Bacillus* sp. and *St. aureus*, but it was found that the ampholytes in this region gave just as much activity when tested as a control. Weak lysozyme activity was detected in the material eluted, none in the ampholyte control.

3. Characterization of the antibacterial components

(i) Susceptibility to proteolysis

Lysozyme activity was reduced from 2 mm clearing to zero by exposure to chymotrypsin. It was reduced to 1.5 mm clearing by exposure to boiled chymotrypsin.

Table 1

Lytic activity¹⁾ of (a) yeast extracellular substance and (b) egg-white lysozyme (0.1 mg/ml) after heating for various periods of time at various temperatures

Lytische Aktivität der Substanz aus dem Kultursubstrat (a) verglichen mit Eiweiß-Lysozym (b) nach unterschiedlich langem und hohem Erwärmen

Time heated (min)	Temperature (°C)					
	35	45	55	65	80	100
a) Yeast lytic activity ²⁾						
5	2.0	2.0	2.0	1.0	0.75	0.75
10	2.0	2.0	2.0	1.0	0.75	0
20	2.0	2.0	2.0	1.0	0.50	0
40	2.0	2.0	1.5	0.75	0.25	0
60	2.0	2.0	1.5	0.75	0.25	0
b) Egg-white lysozyme ³⁾						
5	8.0	6.0	6.0	5.0	5.0	4.0
10	8.0	6.0	6.0	5.0	1.5	1.5
20	8.0	6.0	6.0	6.0	0.5	0
40	8.0	6.0	6.0	6.0	0.5	0
60	8.0	6.0	6.0	5.0	0	0

¹⁾ Lytic activity is expressed as the extent of the clearing (mm) from the edge of the well in an agar well assay.

²⁾ Sample before heating gave a clearing of 2.0 mm.

³⁾ Sample before incubation gave a clearing of 8.0 mm.

Activity against *E. coli* was not affected by chymotrypsin. However, it was reduced from a clear zone of 1 mm to zero by exposure to actinidin. It was reduced to 0.75 mm by exposure to boiled actinidin.

(ii) Heat stability

The effect of heat on the stability of the yeast lysozyme activity is shown in Table 1 a. For comparison the effect on egg-white lysozyme treated in the same way is shown in Table 1 b.

There was no reduction seen in the 1.5 mm clear zone on *E. coli* plates with any of the heat treatments of the cell extract preparation.

(iii) Optimum pH for activity

Lysozyme activity was seen between pH 4.0 and pH 9.0, being greatest between pH 6.0 and pH 8.0. Similar results were found with egg-white lysozyme tested in the same way for comparison. There was no change in the pH of the test plates over the period of incubation.

Results from the testing against *E. coli* were complicated by the changes in pH that occurred over the period of incubation: data are therefore given as follows, with the pH of the plates at the end of incubation in brackets. Activity could not be assessed below pH 4.4 and above pH 9.2, inclusive, because no growth at all occurred on these plates. Activity was found to decrease steadily from a clearing of 1 mm at pH 4.8 (5.0) to 0.5 mm at pH 7.8 (8.6), but a clearing of 1.5 mm was seen at pH 8.0 (8.8) and 1.0 mm at pH 8.3 (9.0). There was no clearing at pH 8.6 (9.2).

(iv) Activity against lactic acid bacteria

The results of testing the antibacterial fraction of yeast cell extract obtained from ion exchange chromatography against a variety of bacterial strains used for malolactic

Table 2

Sensitivity of lactic acid bacteria to the antibacterial fraction of yeast cell extract obtained from ion exchange chromatography and to egg-white lysozyme (0.1 mg/ml). Antibacterial activity is expressed as the extent of the clear zone (mm) from the edge of the well in an agar well assay

Die Empfindlichkeit von Milchsäurebakterien gegenüber der bakteriziden Fraktion des Hefezell-extraktes verglichen mit Eiweißlysozym. Die bakterizide Aktivität ist ausgedrückt als Größe der Hemmzone im Agrardiffusionstest

Bacterial strain	Activity of yeast extract	Activity of lysozyme
<i>Lactobacillus brevis</i> (L199)	0.5	0
<i>Lactobacillus brevis</i> (L247)	2	0
<i>Lactobacillus casei</i> (L190)	0	0
<i>Lactobacillus casei</i> (L192)	0	0
<i>Lactobacillus hilgardii</i> (L248)	2	0
<i>Leuconostoc mesenteroides</i> (L213)	2	0
<i>Leuconostoc oenos</i> (L205)	3	0
<i>Leuconostoc oenos</i> (L240)	0	0
<i>Leuconostoc oenos</i> (L242)	0	0
<i>Pediococcus cerevisiae</i> (L221)	0	0
<i>Bacillus</i> sp. (control)	4	3
<i>Escherichia coli</i> (control)	1.5	<0.5
<i>Staphylococcus aureus</i> (control)	5	2

fermentation in wine-making are shown in Table 2. For comparison, the effect of egg-white lysozyme on these strains is also shown.

Discussion

The antibacterial substances isolated and partially characterized in this study may not be the only ones produced by the yeast, but even if so, they are probably the major ones. The low level of activity found in the cell extract and extracellular medium and the relative insensitivity of the agar-well diffusion method of assay (JAMES *et al.* 1972) could have allowed other antibacterial substances to go undetected by passing through the ion exchange column when the ones isolated were concentrated by being bound to the column. Any substances missed in this way would have to be uncharged or strongly acidic to pass through the column at pH 3. Any significant quantity of less strongly acidic antibacterial material present would probably have been detected in at least the earlier fractions of the frontal elution by the phosphate buffer at neutral pH.

The use of ion exchange to recover the antibacterial substances in a concentrated form was adopted as a result of the findings from preliminary investigations. It was also because of the discovery of the cationic nature of the substances that the procedure was adopted of leaving the disintegrated cells stand with H_2SO_4 added; this was found to help release cationic antibacterial proteins from minced animal tissue (BLOOM and PRIGMORE 1952). It is possible that the antibacterial substances recovered after this could have been formed by acid hydrolysis of larger proteins, but this seems unlikely in view of the finding of proteins with the same characteristics in the extracellular medium to which sulphuric acid was not added. Although most of the investigations reported in this study were carried out on cell extracts, in the earlier part of the study the extracellular medium was also investigated with similar results. The cell extract was used preferentially because much larger volumes of extracellular medium had to be processed through the ion exchange column to recover enough active material for study.

The elution of the active material over such a large number of fractions from the ion exchange column made difficult the purification and characterization of the antibacterial substances. Although the spread could have been due to the NaOH not being strong enough to properly displace the strongly cationic substances, the obvious heterogeneity seen in the antibacterial activity on gel filtration chromatography is more likely to be the cause. The heterogeneity could have been the result of aggregation/disaggregation of the substances during chromatography; this is a feature of strongly cationic antibacterial proteins of mammalian origin (SHANNON *et al.* 1987). This possibility is indicated by the marked tailing observed on electrophoresis without SDS but not with SDS. Egg-white lysozyme has been found to form dimers and higher oligomers under certain conditions (HAMPE 1972). This could also count for the diffuse band, sometimes discernible as a double band, seen on electrophoresis in the region where lysozyme-like activity was detected.

The occurrence of the antibacterial proteins in aggregated forms is also indicated by the findings in SDS electrophoresis. This diaggregating technique gave fewer bands than were found on cationic electrophoresis, and all were of lower molecular weight (14 kDa or lower) than those eluted at the void volume of the column of Sephadex G50 (molecular weight 30 kDa or more).

Although attempts were made to recover bands from the gel after cationic electrophoresis to run on SDS electrophoresis, no data were obtained on the molecular weight of the bands from cationic electrophoresis. Because no assays of antibacterial activity could be carried out with SDS present, neither was it possible to determine the molecular weight of the substances responsible for each type of antibacterial activity.

Even though the antibacterial substances could not be characterized, it was clearly indicated that there are two different types of antibacterial activity produced by the yeast. Although the two types were not satisfactorily separated by column chromatography, fractions were obtained which had no detectable lysozyme-like activity but which had good activity against *E. coli* and *St. aureus*, both species unaffected by egg-white lysozyme at levels that could be detected by the assay used. Also, cell extracts had high activity against *Bacillus* sp. but usually had low or no lysozyme-like activity, whereas in the extracellular medium it was the inverse of this.

Further characterization would be necessary before it could be stated that this activity is lysozyme. Dissolution of bacterial cell walls can be brought about by proteases as well as lysozyme (GHUYSEN *et al.* 1966). However, this activity studied does have similarities to lysozyme in its heat stability, pH optimum, isoelectric point and electrophoretic mobility. Also, there is the suggestion that it is of similar molecular weight to lysozyme, but the band corresponding with this molecular weight obtained on SDS electrophoresis could not be tested.

Inability to test for activity after both forms of electrophoresis and after isoelectric focussing made it impossible to draw definite conclusions about the characteristics of the other type of antibacterial substance produced by the yeast. Unless it was a very minor component of the cationic fraction of the yeast cell extract, then it was presumably one or both of the bands of low molecular weight seen on SDS electrophoresis and one of the two bands of high pI found consistently on isoelectric focussing. That this substance was in fact of low molecular weight is indicated by antibacterial activity being in the third peak (eluted near the bed volume) obtained on gel filtration chromatography. If it were also the substance that gave the band of high mobility on cationic electrophoresis, it would be in all respects similar to the antibacterial peptide found by SHANNON *et al.* (1987) in bovine seminal plasma; this peptide also has high thermal stability and occurs in the form of aggregates.

Whatever the nature of the antimicrobial substance may be, it appears to be distinct from the killer factors produced by some strains of *S. cerevisiae*. These factors do not kill killer strains of *S. cerevisiae* in the same group (YOUNG and YAGIU 1978), yet when a killer strain (A820BK) and a sensitive strain (S14a) (both kindly supplied by H. BUSSEY, MCGILL University) were tested with the cationic preparation from the cell extract of *S. cerevisiae* R107, both strains were killed. As egg-white lysozyme had no activity against these strains and the preparation from the extracellular medium had very little, it was presumed to be the factor other than the lysozyme-like one that was responsible for killing the yeasts. It was also presumed to be this factor responsible for the activity of the cell extract preparation in killing strains of the lactic acid bacteria, as these species were not affected by egg-white lysozyme at 0.1 mg/ml.

This raises the question whether the level of antibacterial activity in wine after primary fermentation is likely to be high enough to affect malolactic fermentation, as this non-lysozyme antibacterial activity was found more in the cell extract than in the extracellular medium. However, the agar-well assay used is the least sensitive assay of antibacterial assay. JAMES *et al.* (1972) found that no inhibition could be detected by

this technique under the same conditions that gave completely inhibition in liquid culture. Without the dilution into the agar in the assay the activity could well be high enough in the ferment to cause some inhibition. Also, the activity detected after concentration in the ion exchange procedure varied from batch to batch. When the inoculum of *S. cerevisiae* R107 was taken fresh from wine fermentation rather than laboratory sub-cultures, even higher levels of activity were obtained. Thus this substance could be responsible for the difficulties sometimes experienced in establishing malolactic fermentation.

It is possible that higher levels of antibacterial activity could occur in the extracellular medium if yeast cells lyse at the end of the primary fermentation. For this reason it could be advantageous to commence the malolactic fermentation earlier.

It may be that successful establishment of malolactic fermentation could more readily be achieved if more regard could be given to the choice of yeast strain; in our preliminary studies differences were found between strains in their production of antibacterial factors. Likewise in the choice of strains of lactic acid bacteria regard may need to be given to differences in susceptibility to these factors.

Summary

Substances with antibacterial activity were recovered from the extracellular medium and from the acidified cell extract of *Saccharomyces cerevisiae* R107 by binding onto a cation exchanger. The fraction eluted with NaOH contained two different antibacterial factors, one with the characteristics of lysozyme, the other apparently a small protein with a similar high pI.

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Correspondence to:

Dr. P. C. MOLAN
 Department of Biological Sciences
 University of Waikato
 Private Bag 3105
 Hamilton
 New Zealand