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Proteolytic Enzymes of *Saccharomyces carlsbergensis*

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1. Of four proteolytic enzymes isolated from autolysing *Saccharomyces carlsbergensis*, one is inactivated at about 45°C, whereas the others are stable at 50°C. pH optima for activity are from 3.0 to 8.0 but maximum stability is between pH 6.0 and 6.5. All appear to be glycoproteins, the carbohydrate moiety containing glucose and mannose residues. 2. Lysed protoplasts of the same yeast release four proteolytic enzymes each of which have two pH optima at pH 3.0 and 7.0 approximately. Compared with the enzymes from autolysed yeast, resistance to high temperature is much less, and they are not glycoprotein in nature. 3. The same yeast grown with *N*-acetyltyrosine ethyl ester as nitrogen source secretes into the medium four proteases believed to be glycoprotein in nature. Generally they resemble the enzymes from lysed protoplasts more than those from autolysing yeast.

Autolysis is important in commercial yeast extract production and in the isolation of biochemically important enzymes, nucleotides, etc. Nevertheless little attention has been paid to biochemical details of autolysis, such as the role played by proteolytic enzymes. Two proteases liberated by autolysis of *S. cerevisiae* with chloroform, having optimum pH values for activity of 3.7 and 6.2 respectively, were described by Dernby (1918). The former contrasted with the latter in being extremely labile in urea solutions and having no essential thiol groups. Two enzymes of autolysed yeast with the same pH optima were purified by Lenney & Dalbec (1967), and Hata, Hayaishi & Dor (1967) described the purification of three such enzymes.

The proteolytic enzymes of living yeast have also received little attention, especially extracellular enzymes such as those demonstrated by Morris & Hough (1956). The purpose of the present paper is to compare the proteases from autolysing yeast, lysed protoplasts of yeast and extracellular secretions from whole living yeast cells.

EXPERIMENTAL

Methods

Micro-organisms and media. Stock cultures of *S. carlsbergensis* N.C.Y.C. 748 (from the National Collection of Yeast Cultures, the Brewing Industry Research Foundation, Nutfield, Redhill, Surrey, U.K.) were maintained at 30°C in a liquid semi-synthetic medium (3g of yeast extract, 3g of malt extract, 5g of bacterial peptone and 10g of glucose/l of water). Synthetic media were based on that of Cutts & Rainbow (1950). Large quantities of yeast were grown at 16–17°C in all-malt wort (sp.gr. 1.040) in a stainless-steel fermenter (55 litres).

Chemicals. Yeast extract, malt extract and bacterial peptone used in the semi-synthetic medium were obtained from Oxoid Ltd., London E.C.4, U.K. Reagents were of analytical-reagent grade or the purest grade available from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K. Sources of fine chemicals were as follows: casein (soluble, light, white) from Hopkin and Williams Ltd; Folin–Ciocalteau reagent, Cyanogum 4L and 2-dimethylaminoethyl cyanide from British Drug Houses Ltd., Poole, Dorset, U.K.; haemoglobin, TEAE-cellulose (triethylaminoethylcellulose), DEAE-Sephadex, *p*-chloromercuribenzoate and *N*-acetyltyrosine ethyl ester from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.; di-isopropyl phosphorofluoridate and Aquacide from Koch–Light Ltd., Colnbrook, Bucks. U.K.; Naphthalene Black 10B and Basic Fuchsin from George T. Gurr Ltd., London S.W.6, U.K. Sephadex was obtained from Pharmacia, Uppsala, Sweden.

Buffers. All buffers were prepared as described by Gomori (1955).

Measurement of proteolytic activity. Measurement of proteolytic activity was based on the appearance of peptides with either 1% (w/v) casein or 1% (w/v) acid-denatured haemoglobin as substrate (Anson, 1938). Peptide content was determined by either the Folin–Ciocalteau reagent (Lowry, Rosebrough, Farr & Randall, 1951) or by direct measurement of E^{1cm}_{280}. A standard curve for tyrosine was used for calibration purposes and activity was expressed as nmol of tyrosine produced/h per mg of protein.

Determination of protein. This was essentially the method of Lowry *et al.* (1951) with casein as a standard.

Determination of carbohydrates. Total carbohydrate

was determined by the method of Ghosh, Charalampous, Sison & Borer (1960). With mixtures of glucose and mannose, individual sugar contents were determined as described by McMurrough & Rose (1967). The hydrolysis of glycoproteins was carried out by the method of Spiro (1966). Identification of neutral sugars followed the procedure of Candy (1967).

Ion-exchange chromatography. Before use, TEAE-cellulose or DEAE-Sephadex was washed and packed as described by Peterson & Sober (1962). Column operations were carried out at 0–4°C and eluent volumes were assayed for protein and proteolytic activity as described above.

Gel filtration. Sephadex G-25 and G-200 were used according to manufacturer's recommendations. For calibration of eluents from G-200, the following proteins of known molecular weight were used on the column: cytochrome *c*, ovalbumin, serum albumin and chymotrypsin.

Identification of products of enzyme action. With 1% (w/v) casein as substrate, enzyme preparations were incubated for a predetermined time at 25°C and pH 7.0. The reaction was stopped by adding Hg^{2+} to give a final concn. of 5 mM and the solution was applied to a Sephadex G-25 column (0.8 cm × 30 cm). Fractions (1 ml) were collected and E_{280}^{1cm} values measured spectrophotometrically. To get a rough estimate of the molecular weights of the fractions, controls each comprising enzyme plus Hg^{2+}, casein and tyrosine were similarly eluted and monitored spectrophotometrically.

Polyacrylamide gel electrophoresis. The method was based on that of Akroyd (1967). Protein was stained with a 1% (w/v) solution of Naphthalene Black in water–methanol–acetic acid (5:3:1, by vol.) for 2 min and excess of stain removed by repeated washing in aq. 7% (v/v) acetic acid. Carbohydrate was stained by the method of Hotchkiss (1948).

Ultracentrifugal analysis. A Beckman–Spinco model E analytical ultracentrifuge was used; the sample was placed in a double-sector synthetic-boundary cell and viewed at 42040 rev./min.

Optimum pH *and temperature for enzyme activity.* The enzymes were prepared as described below under 'Enzyme preparations.' Haemoglobin in 0.06 M-HCl was dialysed against the appropriate buffer and the enzyme added (approx. 5 mg), and held for 1 h. Reaction was stopped by adding 10% (w/v) trichloroacetic acid. For optimum pH the temperature was 25°C, for optimum temperature the pH was the optimum for the particular enzyme.

Activators and inhibitors of enzyme activity. The same system as above was used at 25°C at the optimum pH for the particular enzyme. The enzyme was mixed with the activator or inhibitor for 15 min at 25°C before the substrate was added; the final concn. of activator or inhibitor was 5 mM.

Enzyme preparations

Preparation of proteases by autolysis. This was based on the method of Hata *et al.* (1967).

Stage 1: autolysis and preparation of crude extract. Freshly harvested yeast (1 kg wet wt.) was autolysed by suspension in chloroform (400 ml) at 25°C for 1 h. An equal volume of water was then added, the pH adjusted to 7.0 with 1 M-NaOH and the suspension held overnight at 25°C. After centrifuging for 30 min at 3000*g*, the supernatant liquid was adjusted to pH 5.0 with 1 M-HCl and kept overnight under toluene at 25°C. If protein precipitated, it was removed by centrifugation.

Stage 2: purification of protein. $(NH_4)_2SO_4$ was added to the extract to 90% saturation (660 g/l) at pH 5.0 and the precipitate separated by centrifugation at 24000*g* for 30 min. The collected precipitate of total protein was dissolved in the minimal amount of water and dialysed overnight against 0.01 M-sodium phosphate buffer, pH 7.0, containing 0.1 M-NaCl. Any precipitate in the non-diffusible material was separated by centrifugation.

Stage 3: chromatography on TEAE-cellulose. The non-diffusible material after dialysis was concentrated to approx. 30 ml with Aquacide (a CM-cellulose derivative) at 4°C overnight, and then redialysed as in Stage 2. It was then applied to a TEAE-cellulose column (3.8 cm × 25 cm) at 4°C, previously equilibrated with the same buffer as the dialysing solution. Protein was eluted by increasing the concentration of NaCl in the buffer from 0.1 M to 0.5 M in steps of 0.025 M each (total vol. approx. 100 ml).

Stage 4: chromatography on DEAE-Sephadex. From the TEAE-cellulose column, the fractions showing proteolytic activity were combined and concentrated with Aquacide at 4°C, overnight. After dialysis as in Stage 2, the solution was applied to a DEAE-Sephadex column (0.8 cm × 12 cm). Protein was eluted as in Stage 3.

Preparation of yeast protoplasts. Based on the method of Eddy & Williamson (1957), yeast (about 1.5 mg dry wt./ml) was treated with snail gut juice [final concn. 2% (w/v)], to which had been added 0.1 vol. of 1% (w/v) cysteine hydrochloride. Snail gut juice was 'Suc d'*Helix pomatia*' from Industrie Biologique Française S.A., 35 à 49, Quai du moulin de cage, Gennevillers, Seine, France. After 3 h incubation at 30°C, the protoplasts were separated by centrifugation at 3000*g* for 5 min and washed three times with ice-cold 5 mM-sodium citrate–phosphate buffer, pH 5.8, containing 0.8 M-mannitol. The yield of protoplasts was greater than 98% on the basis of phase-contrast microscopical examination.

Preparation of proteases from protoplasts. Protoplasts were lysed by injection of a concentrated suspension into 10 vol. of ice-cold water and held at 4°C for 30 min, with occasional stirring. The resulting suspension was centrifuged at 1500*g* for 15 min to remove all membranes and cells resistant to treatment. The supernatant liquid was centrifuged at 24000*g* for 2 h to precipitate protein, which was then washed twice with ice-cold water. The protein was dissolved in a minimum amount of water and dialysed overnight against 0.01 M-sodium phosphate buffer, pH 7.0, containing 0.1 M-NaCl. The protein solution was applied to a TEAE-cellulose column (0.8 cm × 20 cm) and eluted by increasing stepwise the concentration of NaCl in the buffer from 0.1 to 0.5 M, approx. 10 ml being used for each step.

Demonstration of proteases secreted by living yeast cells. A synthetic medium based on that of Cutts & Rainbow (1950) was modified with 3% (w/v) glucose, mannose or a mixture of the two as a carbon source, and casein (400 μg of N/ml) as nitrogen source. Yeast was grown at 30°C in a Gyrotory shaker (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.) for 64 h and separated from the medium by centrifugation at 3000*g* for 10 min. The supernatant liquid was assayed for proteolytic activity.

Preparation of proteases secreted by living yeast cells. A version of the synthetic medium was prepared with 3% (w/v) glucose as carbon source and *N*-acetyltyrosine ethyl ester (100μg of N/ml) as nitrogen source. Yeast was grown and separated as above; the supernatant liquid (500ml) was concentrated to 30ml with Aquacide at 0–4°C and saturated with $(NH_4)_2SO_4$. The resultant precipitate was centrifuged at 24000*g* for 2h. After two washings with a saturated aq. $(NH_4)_2SO_4$ solution, the protein was dissolved in the minimum amount of water and exhaustively dialysed at 0–4°C against 0.01M-sodium phosphate buffer, pH7.0, containing 0.1M-NaCl. The resulting solution was applied to a TEAE-cellulose column and protein eluted by increasing the concentration of NaCl in the buffer from 0.1M to 0.5M.

RESULTS

Proteases from autolysed yeast. In a typical preparation the total protein applied to a TEAE-cellulose column was approx. 10g, the specific activity at 25°C and pH7.0 being approx. 5.0nmol of tyrosine produced/h per mg of protein. When selected, pooled fractions amounting to 6g of protein with a specific activity of 7.0 (see the Experimental section) were applied to a DEAE-Sephadex column, the resulting new fractions were pooled and designated as shown in Fig. 1. The specific activities for the four fractions A, B, C and D were 12, 30, 20 and 22nmol of tyrosine/h per mg of protein respectively. Fraction A is the most abundant but is the least stable. Electrophoresis of each fraction on polyacrylamide gel produced only one band, suggesting that the four fractions were single proteins. In confirmation, ultracentrifugal analysis displayed only one peak for each fraction. The optimum pH for proteolytic activity at 25°C on 1% (w/v) acid-denatured haemoglobin (Fig. 2) was 8.2, 6.6, 6.3 and 2.9 for fractions A, B, C and D respectively. The four fractions were most stable at pH6.0–6.5. The optimum temperatures for proteolytic activity of each fraction (Fig. 3) were 30–35°C, 50°C, 50°C, 60°C for fractions A, B, C and D respectively. The stability of each fraction at various temperatures was measured (Fig. 4). Fraction A is increasingly unstable as temperature rises whereas fractions B, C and D are stable at much higher temperatures. With casein as substrate, fraction A produced amino acids or peptides of low molecular weight in less than 1h. The products of digestion over the same period with fractions B, C and D had molecular weights of over 5000. It therefore seems that A is an exopeptidase and the others are endopeptidases. The effects of selected activators and inhibitors on the fractions were studied (Table 1) and the inhibition of fraction A by di-isopropyl phosphorofluoridate and of fraction B by *p*-chloromercuribenzoate are noteworthy. Analysis of the fractions (Table 2) revealed

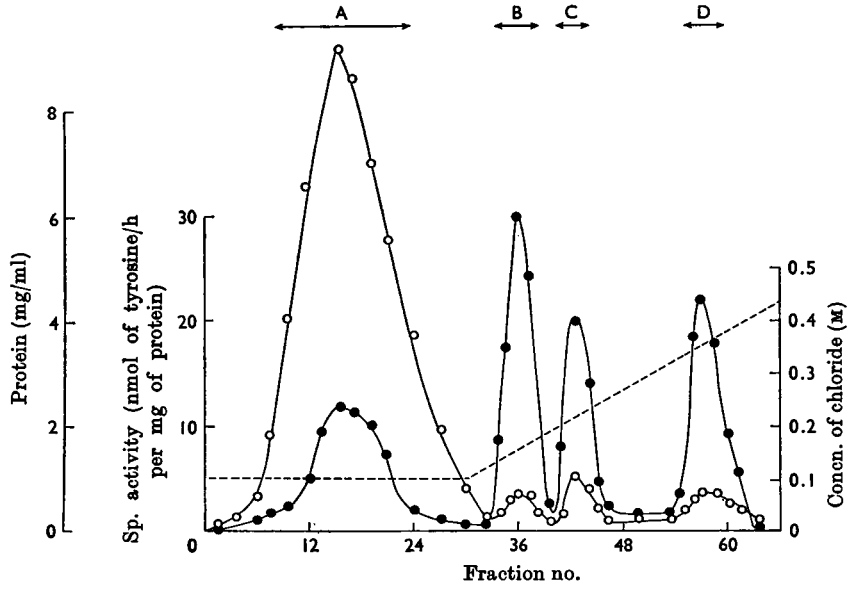

Fig. 1. Typical elution pattern from a DEAE-Sephadex column. Protein was eluted by stepwise increases in the concentration of NaCl in the buffer from 0.1M to 0.5M. Fractions (3ml) were collected. Activities were measured in duplicate with a 1% acid-denatured haemoglobin solution, at 25°C and pH7.0. ○, Protein; ●, specific activity; ----, concn. of NaCl.

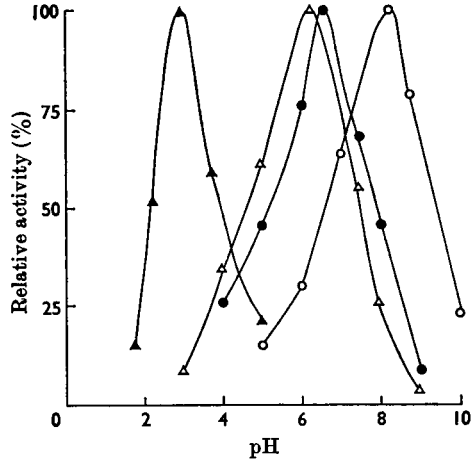

Fig. 2. pH optima of preparations from autolysed yeast. Activity was measured in duplicate at 25°C with a 1% acid-denatured haemoglobin solution as substrate. ○, Enzyme A; ●, enzyme B; △, enzyme C; ▲, enzyme D.

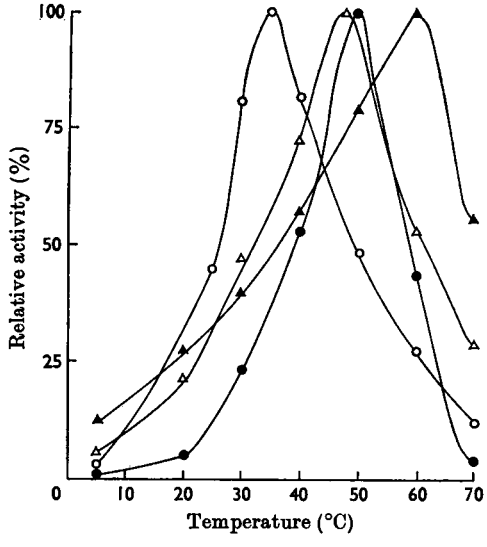

Fig. 3. Temperature optima of preparations from autolysed yeast. Activity was measured in duplicate with a 1% acid-denatured haemoglobin solution as substrate. The pH values were: A, pH 8.2 (○); B, pH 6.6 (●); C, pH 6.3 (△); D, pH 2.9 (▲).

the presence of carbohydrate moieties, which hydrolysed to give glucose and mannose. Electrophoresis of each of the fractions on polyacrylamide gel and subsequent staining showed one carbohydrate band which had the same mobility as the protein

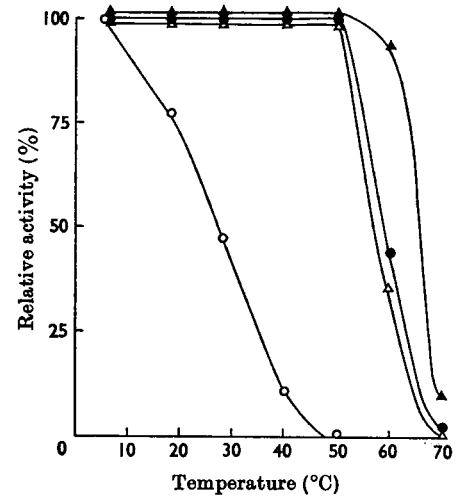

Fig. 4. Stability to temperature of preparations from autolysed yeast. Each enzyme was incubated at the set temperature at pH 6.5 for 1 h, and the activity then measured in duplicate at 25°C at the stated pH value: A, pH 8.2 (○); B, pH 6.6 (●); C, pH 6.3 (△); D, pH 2.9 (▲).

Table 1. *Effects of various activators and inhibitors on the proteolytic enzyme fractions from autolysed yeast*

The assays were performed in duplicate with a 1% (w/v) casein solution as substrate, at 25°C for 1 h. The pH values were 8.0, 6.5, 6.5 and 3.0 for A, B, C and D respectively.

Inhibitor (5 mM final concn.)	Remaining activity of fractions (%) A	B	C	D
None	100	100	100	100
Di-isopropyl phosphorofluoridate	0	100	100	100
p-Chloromercuribenzoate	100	0	100	100
Cysteine	100	135	100	100
Ca^{2+}	85	170	100	100
Mg^{2+}	130	130	120	100
EDTA	100	100	65	100
Hg^{2+}	0	0	5	0

Table 2. *Carbohydrate content of the proteolytic enzyme fractions from autolysed yeast*

	Fractions A	B	C	D
Carbohydrate/carbohydrate +protein ratio (as %)	7	4	20	13
Glucose:mannose ratio	1:1	2:1	2:1	1:2

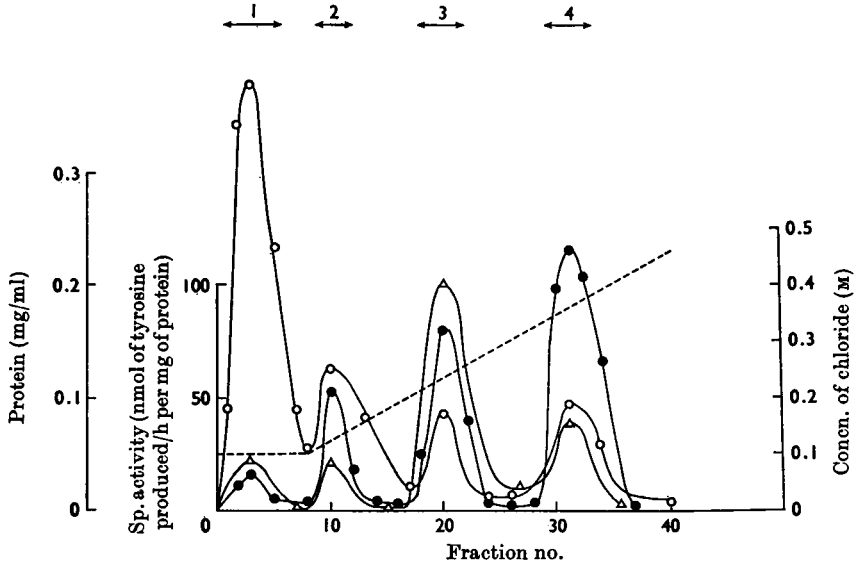

Fig. 5. Typical elution pattern from a TEAE-cellulose column of the protoplast preparation. Protein was eluted by increasing the concentration of NaCl in the buffer from 0.1 M to 0.5 M. Fractions (3 ml) were collected. Activities were measured in duplicate on a 1% casein solution at 25°C. ○, Protein; ●, specific activity at pH 7.0; △, specific activity at pH 3.0; ----, concn. of NaCl.

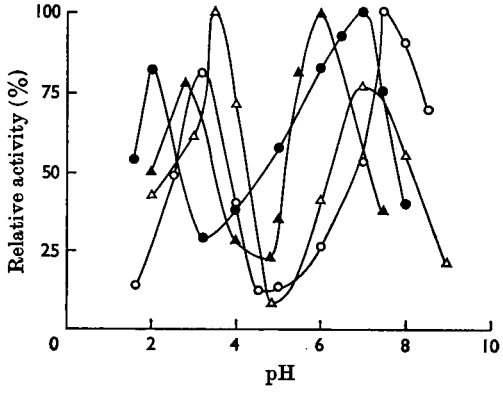

Fig. 6. pH optima of preparations from protoplasts. Activity was measured at 25°C in duplicate with a 1% casein solution as substrate. ○, Enzyme 1; ●, enzyme 2; △, enzyme 3; ▲, enzyme 4.

moiety, suggesting that the fractions are glycoprotein. Applied to a Sephadex G-200 column, the carbohydrate and protein moieties were eluted simultaneously. By comparison with standard proteins, the estimates for molecular weight were 60000±5000 for fraction A and 75000±5000 for the others.

Proteases from protoplasts. The total protein obtained from 140 mg dry wt. of yeast cells and 80 mg dry wt. of derived protoplasts was approx. 10 mg. The specific activity of this protein was approx. 84 nmol of tyrosine produced/h per mg of protein at 25°C and pH 7.0. A typical elution pattern from a TEAE-cellulose column (Fig. 5) shows the presence of four fractions (designated 1, 2, 3 and 4). Each fraction was free of carbohydrate. The pH optimum for the proteolytic activity of each fraction was measured at 25°C on a 1% (w/v) casein solution. Results in Fig. 6 show two pH optima for each fraction, one being about pH 3.0 and the other at approx. 7.0 The specific activities of the fractions are given for both pH values. No fraction was as stable to high temperatures (Fig. 7) as the corresponding fractions from autolysed yeast. The most noteworthy effect of various activators and inhibitors (Table 3) is the stimulation of activity of each fraction by Zn^{2+} at pH 7.0. The fact that both EDTA and metal ions can stimulate activity suggests that the metal ion is not an integral part of the enzyme, in our preparation. The products of proteolysis were eluted from a Sephadex G-25 column and are consistent with the preparations obtained from autolysed yeast. Thus fraction 1 produced amino acids or peptides of low molecular weight whereas the other three gave products of molecular weight over 5000. Determinations of the molecular weights of fractions by using Sephadex

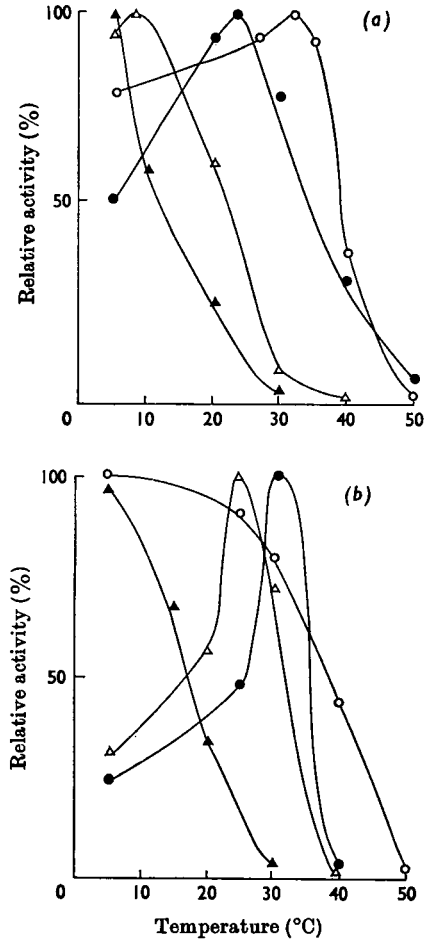

Fig. 7. Temperature optima of preparations from protoplasts. Activity was measured in duplicate at (*a*) pH 7.0, or (*b*) pH 3.0, with a 1% casein solution as substrate. ○, Enzyme 1; ●, enzyme 2; △, enzyme 3; ▲, enzyme 4.

G-200 were 20 000 ± 5000 for fraction 1, 70 000 ± 5000 for fractions 2 and 3 and 80 000 ± 5000 for fraction 4. During protoplast preparation some proteolytic activity was released into the medium and increased with time. The total activities associated with the intact yeast cells, the derived protoplasts and that released into the medium were determined as 160, 30 and 800 nmol of tyrosine produced/h respectively. To investigate whether protoplasts were synthesizing new proteolytic enzymes, protoplasts were incubated at 30°C, with occasional shaking, in a medium comprising 0.1% casein and 0.8 M-potassium chloride, with or without 0.02 M-glucose (Fig. 8). The total activity and amount secreted into the medium was measured.

Table 3. *Effects of various activators and inhibitors on the proteolytic enzyme fractions from protoplasts*

(*a*) Assays were performed in duplicate on a 1% (w/v) casein solution at 25°C and pH 7.0 for 1 h.

	Remaining activity (%)			
Fractions ...	1	2	3	4
Inhibitor (10 mM final concn.)				
None	100	100	100	100
Di-isopropyl phosphorofluoridate	100	100	100	0
p-Chloromercuribenzoate	0	100	100	100
Cysteine	200	100	100	30
Ca^{2+}	60	110	125	0
Mg^{2+}	95	150	150	100
Zn^{2+}	300	300	400	120
EDTA	0	500	100	160

(*b*) Assays were performed in duplicate on a 1% (w/v) casein solution at 25°C and pH 3.0 for 1 h.

	Remaining activity (%)			
Fractions ...	1	2	3	4
Inhibitor (10 mM final concn.)				
None	100	100	100	100
Di-isopropyl phosphorofluoridate	0	100	100	0
p-Chloromercuribenzoate	0	100	20	0
Cysteine	25	0	50	50
Ca^{2+}	0	200	100	100
Mg^{2+}	0	180	100	0
Zn^{2+}	50	180	20	50
EDTA	25	500	30	300

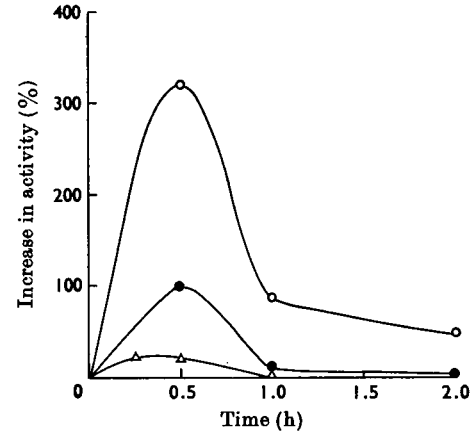

Fig. 8. Increase in proteolytic activity of protoplasts during incubation in an osmotically buffered protein medium at 30°C. Activity was measured in duplicate at pH 7.0 and 25°C on a 1% casein solution. ○, Medium contains 0.02 M-glucose (activity of protoplasts + medium); ●, medium contains no glucose (activity of protoplasts + medium); △, medium contains no glucose (activity of medium).

Table 4. *Effect of carbon and nitrogen constituents in the medium on the secretion of proteolytic activity into the medium, and the amount of protein obtained from autolysed yeast*

Activity was measured in duplicate with a 1% (w/v) casein solution as substrate at pH 7.0 and 25°C, and expressed as nmol of tyrosine produced/h per ml of medium. The yeast was autolysed as described in the Experimental section.

		Nitrogen source		
	Carbon source	Casein hydrolysate	$(NH_4)_2SO_4$	Casein
Glucose	Yeast growth (mg dry wt./ml)	5.0	4.4	0.5
	Proteolytic activity	—	—	1.2
	Protein (mg/g of autolysed yeast)	7.5	7.4	21.0
Glucose + mannose (1:1)	Yeast growth (mg dry wt./ml)	5.0	4.4	0.6
	Proteolytic activity	—	—	8.4
	Protein (mg/g of autolysed yeast)	15.0	9.4	34.5
Mannose	Yeast growth (mg dry wt./ml)	5.0	4.4	0.5
	Proteolytic activity	—	—	1.2
	Protein (mg/g of autolysed yeast)	7.3	6.7	20.2

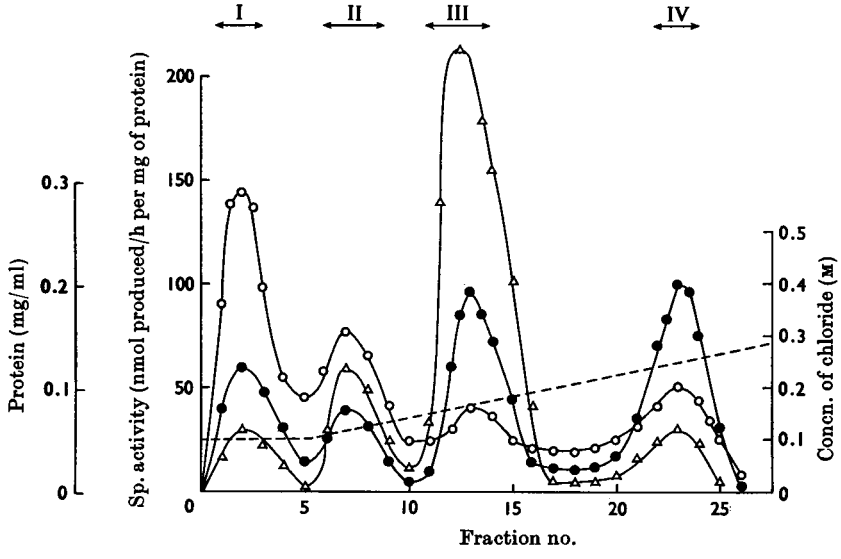

Fig. 9. Typical elution pattern from a TEAE-cellulose column of the preparation secreted by living yeast. Protein was eluted by increasing the concentration of NaCl in the buffer from 0.1 M to 0.5 M. Fractions (3 ml) were collected. Activities were measured in duplicate on a 1% casein solution at 25°C. ○, Protein; ●, specific activity at pH 7.0; △, specific activity at pH 3.0; ----, concn. of NaCl.

Proteases secreted extracellularly by living yeast cells. Yeast grown with casein as sole nitrogen source secretes proteolytic enzymes to a small extent into the medium. Table 4 shows that with ammonium sulphate and casein hydrolysate there is no comparable secretion. When glucose and mannose mixture was the carbon source the secretion of proteolytic enzymes and the total cell

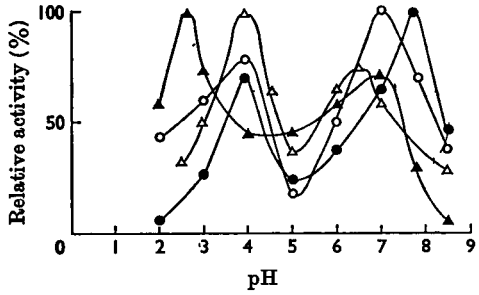

Fig. 10. pH optima of preparations from living yeast. Activity was measured in duplicate at 25°C with a 1% casein solution as substrate. ○, Enzyme 1; ●, enzyme II; △, enzyme III; ▲, enzyme IV.

Table 5. *Carbohydrate content of the fractions showing proteolytic activity that are secreted by living yeast cells*

	Fractions			
	I	II	III	IV
Carbohydrate/carbohydrate+protein ratio (%)	38	2	8	22
Glucose : mannose ratio	3:1	100% Mannose	1:3	1:3

proteins was increased. The secretion was inhibited by neither amino acids nor proline in the presence of protein. *N*-acetyltyrosine ethyl ester is able to induce proteolytic activity and yield a total protein secretion of approx. 4.5mg from 500ml of spent medium. The specific activity was approx. 60nmol of tyrosine produced/h per mg of protein at 25°C and pH7.0. This preparation was applied to a TEAE-cellulose column and a typical elution pattern (Fig. 9) shows the presence of four fractions. Analysis of the fractions indicated that they were glycoproteins (Table 5). The pH optima for proteolytic activity were 3.0 and 7.0 at 25°C on 1% (w/v) casein solution (Fig. 10) and thus indicated a resemblance to the proteases from protoplasts. The effects of temperature (Fig. 11) also show resemblances to proteases from protoplasts rather than to those of autolysing yeast. Among the effects of various activators and inhibitors (Table 6), Zn^{2+} seems to stimulate proteolytic activity. Products of the reaction were similar to those from protoplasts and autolysed yeast. Thus fraction I produced amino acids and peptides whereas fractions II, III and IV did not. Molecular-weight determinations with Sephadex G-200 gave the approx. values, 50000, 70000, 71000 and 71000 respectively, each ±5000.

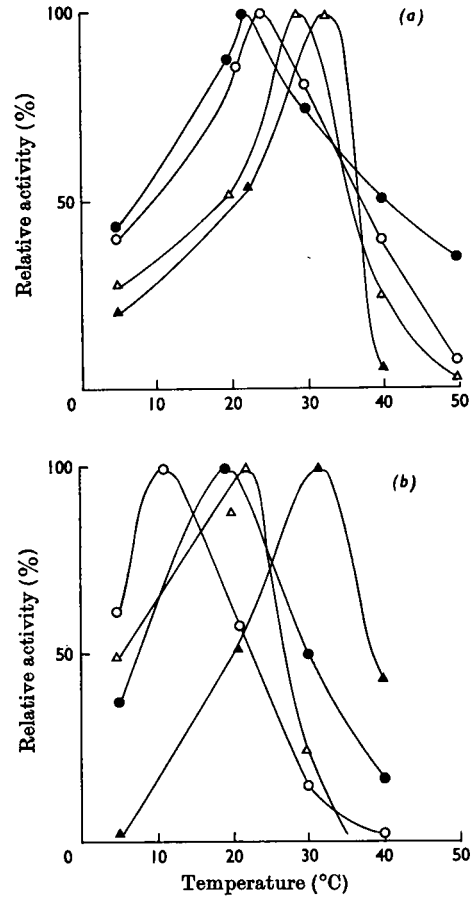

Fig. 11. Temperature optima of preparations from living yeast. Activity was measured in duplicate at (*a*) pH7.0, or (*b*) pH3.0, with a 1% casein solution as substrate. ○, Enzyme I; ●, enzyme II; △, enzyme III; ▲, enzyme IV.

DISCUSSION

In yeast autolysis, four proteolytic enzymes are present; they give a range of products the proportions of which differ according to the conditions of temperature and pH. The best pH value for yeast extract manufacture is 6.0–6.5, which closely agrees with the value at which the proteolytic enzymes that we describe are most stable. Above 45°C, it is industrial experience that the amino nitrogen/total nitrogen ratio decreases and this can now be interpreted in terms of the inactivation of the one protease of autolysing yeast that is capable of producing amino acids. Now that this and other details of the proteases are available, more precise control on the rate of autolysis under industrial conditions becomes possible, and on the quality of the product.

Table 6. *Effects of various activators and inhibitors on the proteolytic enzyme fractions secreted by living yeast cells*

(*a*) Assays were performed in duplicate on a 1% (w/v) casein solution at 25°C and pH 7.0

	Remaining activity (%)			
Fractions ...	I	II	III	IV
Inhibitor (10mM final concn.)				
None	100	100	100	100
Di-isopropyl phosphorofluoridate	0	100	0	100
p-Chloromercuribenzoate	0	0	0	0
Cysteine	100	0	0	0
Ca^{2+}	100	0	100	150
Mg^{2+}	150	0	75	150
Zn^{2+}	200	200	100	200
EDTA	0	0	0	0

(*b*) Assays were performed in duplicate on a 1% (w/v) casein solution at 25°C and pH 3.9.

	Remaining activity (%)			
Fractions ...	I	II	III	IV
Inhibitor (10mM final concn.)				
None	100	100	100	100
Di-isopropyl phosphorofluoridate	0	100	0	100
p-Chloromercuribenzoate	0	0	0	0
Cysteine	150	0	125	150
Ca^{2+}	100	150	125	150
Mg^{2+}	150	150	150	50
Zn^{2+}	175	175	150	175
EDTA	0	0	0	200

Schulze & Colowick (1969) have demonstrated that the conditions of autolysis greatly influence the nature of the hexokinase isolated from yeast. On the basis of their observations they suggest that isoenzymes of yeast hexokinase are artifacts resulting from proteolytic action. This situation may be paralleled in other enzyme preparations involving yeast autolysis and in this connection our results suggest ways to inhibit proteolysis, e.g. low pH or di-isopropyl phosphorofluoridate inhibit the most abundant protease.

There is now evidence that proteolytic enzymes of autolysing yeast are glycoproteins, in common with extracellular enzymes of yeast, other fungi and animals (Lampen, 1968) and in contrast with intracellular enzymes. The linkages between carbohydrate and protein moieties of yeast cell-wall glycoproteins have been discussed by Sentandreu & Northcote (1968) and the presence of similar carbohydrates in the proteolytic enzymes of autolysing yeast suggests that the linkages may be the same.

The proteolytic enzymes secreted by living yeast cells are also glycoprotein but in other respects these enzymes resemble those from lysed protoplasts rather than those from autolysing yeast, e.g. in pH optima and temperature stability. This suggests that the enzymes from autolysing yeast are of different origin from living yeast or protoplasts but that where a cell wall is present, linkage of the enzymes to carbohydrate occurs. The site of linkage may be within the cell wall or in the region just inside the plasma membrane where active synthesis of cell-wall material occurs. With protoplasts, where there is no cell-wall template, active synthesis of cell-wall material does not occur. In the synthesis of liver glycoproteins, carbohydrate is added after polypeptide synthesis (Sarcione, Bohne & Leahy, 1964) and the linkage is associated with the endoplasmic reticulum. Immunoglobulin synthesis appears to be similar; addition of carbohydrate occurs during intracellular transit of the protein (Swenson & Kern, 1968). During yeast budding, Sentandreu & Northcote (1969) noted vesicles accumulating at the site of bud formation and during enlargement of the bud wall. The vesicles may carry material for cell-wall synthesis and in much the same way, intracellular proteolytic enzymes may be transferred through the zone of cell-wall synthesis and to the plasma membrane. The origin of the proteolytic enzymes of autolysing yeast is suggested by Matile & Wienken (1967) from work on vacuoles isolated intact from yeast protoplasts. The vacuoles had complements of proteolytic and other enzymes expected of lysosomes with high specific activities. These enzymes were only released on the approaching death of the cells and could be distinguished from the enzymes secreted extracellularly by their digestion of the mother cell rather than digestion of potential nutrients in the medium.

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