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Three Yeast Proteins That Specifically Inhibit Yeast Proteases A, B, and C

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Baker's yeast was found to contain inhibitors of yeast proteases A and C. These two proteins were partially purified, characterized, and compared with the previously described inhibitor of protease B. The A and B inhibitors were very thermostable and were extracted from intact yeast cells at 95 C. The A inhibitor appeared to be a protein with a molecular weight of about 22,000 which could be dissociated into two monomers or chains, both of which had a molecular weight of approximately 11,000. The protease C (carboxypeptidase Y)-inhibitor complex was purified and then partially dissociated on an ion-exchange column. The free protease C inhibitor was very unstable, possibly because of destruction by a contaminating protease. Each inhibitor was specific for its corresponding protease and each inhibition was competitive. Whereas proteases A, B, and C destroyed the B inhibitor, only protease B had a pronounced destructive effect on the protease A inhibitor. Pepstatin was found to be a selective inhibitor of protease A, whereas chymostatin and antipain specifically inhibited protease B.

There are three well known proteases in *Saccharomyces cerevisiae*: proteases A, B, and C (also known as carboxypeptidase Y). Protease A is an acid protease, whereas proteases B and C are serine proteases.

When proteases A and B were extracted by autolysis at pH 7, it was noted that these enzymes displayed very little activity, but that they could be activated by incubation at pH 5 (18). At that time it was postulated that activation of protease B involved either the conversion of zymogen to active protease, or the destruction of inhibitor present before activation. A similar activation occurs with yeast protease C (14), which was originally thought to be a third endopeptidase, but was subsequently shown to be a carboxypeptidase (10). In 1969 we reported that the inactive form of protease B is an enzyme-inhibitor complex and that yeast autolysates also contain an inhibitor of protease C (20). The present communication presents evidence that the inactive forms of proteases A and C are also enzyme-inhibitor complexes; in addition, the three inhibitors (I_A, I_B, and I_C) were partially purified and characterized. All three proteases are localized within the yeast vacuole, whereas their inhibitors are in the cytosol outside the vacuole (21). A preliminary report of this work has been presented (Fed. Proc. **32**:659, 1973).

MATERIALS AND METHODS

Yeast protease preparations. For most of the inhibitor assays a crude yeast protease preparation containing proteases A, B, and C was used. The proteases were extracted, activated, and fractionated with ethanol as previously described (19). When one of the proteases free of the other two was required, a diethylaminoethyl (DEAE)-Sephadex column separation of the enzymes in the ethanol fraction was made according to Hayashi et al. (13). The 0 to 0.5 M NaCl gradient eluted protease B, then protease A, and finally protease C.

Enzyme solutions were diluted shortly before assay using 5 mM citrate buffer, pH 6.0, containing 0.05% Brij 35SP. This surfactant was found to stabilize these enzymes in dilute solution, as reported for brewer's yeast proteases by Félix and Brouillet (7). The protease A and B assay calibration curves (relating quantity of enzyme to units of enzyme) were not linear; therefore all results were corrected for this nonlinearity as described by Anson (2).

Assay for protease A. Protease A was assayed by a modification of the Anson method (2). Hemoglobin was prepared from beef erythrocytes and was denatured in dilute solution by adjusting to pH 2 with HCl. A 1% solution containing 0.05 M citric acid and 0.02% NaN_3 was stable for several months when stored at pH 2 at 4 C. Portions were adjusted to pH 3.4 with KOH shortly before use. Enzyme solutions contained 0.5 mM *p*-chloromercuribenzoate to prevent interference from proteases B or C. A 0.2-ml sample of the enzyme solution was incubated with 1.5 ml of substrate for 20 min at 30 C. The digestion was stopped by adding 2.5 ml of 0.34 N trichloroacetic acid. To 1.67 ml of the trichloroacetic acid filtrate were added 3.33 ml of 0.3 N NaOH containing 2.9% Na_2CO_3 and then 1.0 ml of diluted Folin-Ciocalteau reagent. After 10 min the blue color was read at 650 nm in a 1-cm cuvette. Blanks were prepared by the same procedure except that the trichloroacetic acid

was added before the enzyme.

Casein substrates at pH 2.5 were similarly used for the analysis of protease A and I_A. However, they produced results which were not nearly as reproducible as those obtained with hemoglobin as a substrate.

In working with protease A and I_A, we avoided borax buffers and high concentrations of sodium ion. Borax (5 mM) or NaCl (0.1 M) in the enzyme solution caused inhibitions of 93 and 27%, respectively. In addition, NaCl lessened the inhibition of protease A by I_A. KCl (0.1 M) had no effect on this enzyme.

Assay for protease B. Protease B was assayed with azocoll as substrate, by using a modification of the method of Juni and Heym (16). Fifteen milligrams of azocoll was suspended in 0.4 ml of 0.1 M phosphate buffer, pH 7.5, containing 0.05% Brij 35SP. A 0.1-ml sample of enzyme was added and the mixture was incubated for 20 min at 30 C with frequent shaking. The reaction was stopped by adding 3.2 ml of 1 mM $HgCl_2$, and then the suspension was centrifuged for 15 min at 12,000 $\times$ g. The optical absorbance of the supernatant solution was measured at 520 nm in a 1-cm cuvette. Blanks were prepared by the same procedure except that the $HgCl_2$ solution was added before the enzyme. We have confirmed the observation of Juni and Heym (16) that protease A and protease C do not act on azocoll.

Units and assay for inhibitors of protease A or B. One unit of protease was defined as the amount required to increase the optical absorbance under these conditions by 1.00. In assaying I_A and I_B, the yeast protease preparation was analyzed with and without added inhibitor, restricting the inhibition to the linear part of the inhibitor calibration curve. One unit of inhibitor was defined as the amount which decreased protease activity by one unit.

Assay of protease C and its inhibitor. Protease C (carboxypeptidase Y) was assayed by using a substrate containing 2.5 mM benzyloxycarbonyl glutamyl-tyrosine (Z-Glu-Tyr) dissolved in 0.05 M acetate buffer, pH 5.0. Enzyme solution (0.06 ml) was incubated with 0.54 ml of substrate for 10 min at 30 C. The amount of tyrosine liberated was measured by using the manual ninhydrin procedure of Moore (26). I_C was assayed by analyzing the yeast protease preparation with and without added inhibitor. The decrease in activity was a measure of the quantity of I_C present (see Fig. 9). The esterase activity of protease C (14) was measured by using a substrate containing 0.01 M *N*-acetyl-L-tyrosine ethyl ester (ATEE) and 0.1 M KCl in a 0.01 M Tris (hydroxymethyl)aminomethane buffer, pH 8.0. Enzyme solution (0.15 ml) was added to 2.0 ml of substrate, and the amount of 0.01 N NaOH needed to titrate the pH to 8.0 was measured at intervals. The milliequivalents of NaOH consumed per minute were a measure of the quantity of protease C present. The protease C·I_C complex was quantitated by activating at pH 4.6 and then analyzing for carboxypeptidase activity. For activation the sample was usually diluted fivefold with a 0.04 M citrate buffer containing 0.1% Brij 35 and 0.001% thymol at pH 4.6. Under these conditions I_C was digested and destroyed during overnight incubation at 25 C.

Assay for protein. The method of Lowry et al. (22) was used, by using bovine serum albumin as a standard. To prevent interference from amino acids and peptides, protein was precipitated with 10% trichloroacetic acid, collected by centrifugation and redissolved in 1 N NaOH before analysis.

Column chromatography. DEAE-cellulose and DEAE-Sephadex A-50 were regenerated and used according to the methods of Peterson and Sober (27). Sephadex was used in molecular weight determinations by using the procedures of Andrews (1) and Whitaker (34).

Materials. The yeast used in this investigation was Fleischmann's compressed baker's yeast (*S. cerevisiae*) which was generously provided by Standard Brands, Inc. Sephadex and DEAE-Sephadex were purchased from Pharmacia; DEAE-cellulose (Cellex-D) from Bio-Rad; Z-Glu-Tyr and ATEE from Cyclo; crystalline ovalbumin, ribonuclease, and pancreatic trypsin inhibitor from Worthington; crystalline trypsin, chymotrypsin, pepsin, and azocoll from Calbiochem; crystalline subtilisin from Nagase; Brij 35SP from Atlas Chemical Industries; other yeasts (*S. carlsbergensis*, *S. fragilis* ATCC no. 10022, and *Candida utilis*) from Sigma.

RESULTS

Effect of hemoglobin renaturation on protease A pH-activity curve. When hemoglobin was denatured by incubation at pH 2.0 and used to determine the effect of pH on the activity of protease A, the upper curve in Fig. 1 was obtained. When hemoglobin substrates were stored at pH 3.0 to 3.7 at 4 C, their digestibility by protease A declined, presumably because the hemoglobin was renaturing slowly. After a substrate had been stored for 2

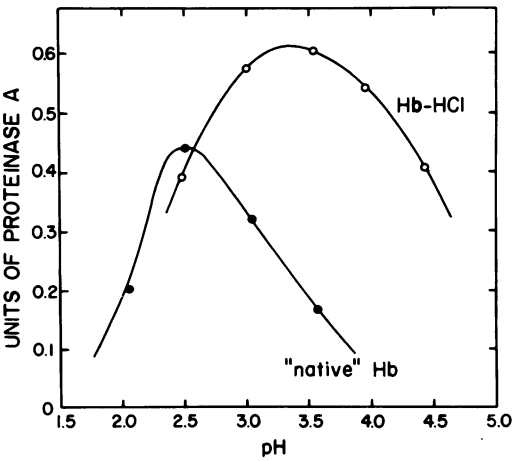

FIG. 1. *The effect of hemoglobin denaturation on the apparent optimum pH of protease A. For the upper curve, hemoglobin freshly denatured at pH 2.0 was used. For the lower curve, hemoglobin which was similarly denatured and then stored for 2 weeks at pH 3.7 at 4 C was used. The same quantity of protease A was used in both experiments. Substrates were buffered with 0.05 M citrate.*

weeks at pH 3.7, it was incubated with the same amount of protease A to again determine the effect of pH on activity. The lower curve in Fig. 1 illustrates that storage at pH 3.7 not only caused a decline in digestibility but also lowered the apparent optimum pH from about 3.4 to 2.6. Therefore, we now store this substrate at pH 2.0 and adjust it to pH 3.4 shortly before use. Under these conditions, substrate digestibility and blank value remain constant for several months. These findings are similar to those of Schlamowitz and Peterson (30) on the effect of substrate denaturation on the apparent optimum pH of pepsin.

Purification of I_A. The partial purification of I_A is summarized in Table 1. One pound of yeast was suspended in 550 ml of water and heated with stirring to 90 C. The suspension was then held at 90 to 95 C for 10 min, cooled, and centrifuged at 1,000 × *g* for 15 min. The supernatant fluid was dialyzed overnight with agitation in cellophane bags versus distilled water at 4 C. During dialysis, I_A was retained, but some of I_B passed through the membrane. KCl (0.05 M) was added to the bag contents and the pH was adjusted to 9.0. This liquid was then passed through a DEAE-cellulose column (4 by 30 cm) which had been equilibrated with a 0.01 M NH_4OH buffer, pH 9.0, containing 0.05 M KCl. When the first protein emerged from the column, the next 460 ml of eluate was collected. This was then evaporated to 50 ml and passed through a column (4 by 85 cm) of Sephadex G-75 equilibrated with a 0.01 M citrate buffer, pH 6.0, containing 0.05% Brij 35. The fractions containing I_A were combined, dialyzed, and then passed through a column (2 by 30 cm) of DEAE-cellulose equilibrated with 0.01 M NH_4OH buffer, pH 9.0. After washing the column with this buffer, the I_A was eluted by using a linear gradient of KCl (0 to 0.3 M). The peak I_A fraction represented a purification of only 22-fold; however, the 95 C extraction step was quite selective because it extracted only about 1% of the yeast protein.

Three other yeasts (*S. carlsbergensis*, *S. fragilis*, and *Candida utilis*) were extracted at 95 C; all were found to contain thermostable inhibitors of *S. cerevisiae* proteases A and B.

Molecular weights of I_A and I_B. The first DEAE-cellulose eluate described above contained both I_A and I_B; a portion of it was chromatographed on a Sephadex G-75 column to determine the molecular weights of these inhibitors. The elution pattern is shown in Fig. 2. The apparent molecular weights were 22,000 for I_A and 9,000 for I_B.

A sample of the second DEAE-cellulose eluate (Table 1, step 4) was subjected to electrophoresis on sodium dodecyl sulfate-acrylamide gel by the method of Weber et al. (33). Figure 3 shows that the I_A band had a molecular weight of about 11,000 and that one faint contaminating protein band was present. These results indicate that I_A consists of two monomers or subunits of equal size. When a low level of I_A was stained with Coomassie brilliant blue, the I_A band showed a pink color instead of the blue color normally seen with other protein bands.

Specificity and protein nature of I_A and I_B. After the separation of I_A and I_B by chromatography on Sephadex (Fig. 2), the I_A fraction did not inhibit protease B and the I_B fraction did not inhibit protease A. In addition, neither inhibited protease C. When I_A and I_B were incubated with highly purified trypsin, chymotrypsin, pepsin, or subtilisin at their respective pH optima, none of these proteases was inhib-

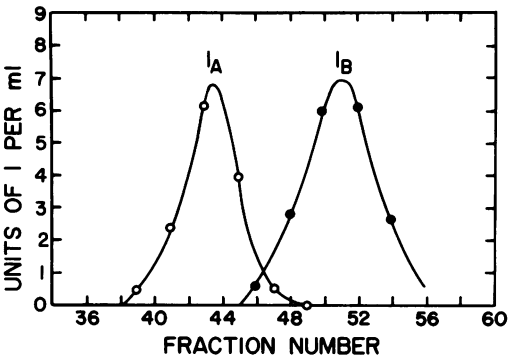

FIG. 2. *Elution pattern of I_A and I_B from a Sephadex G-75 column (1.5 by 115 cm). The column was calibrated using ovalbumin, chymotrypsin, ribonuclease, and panacreatic trypsin inhibitor as standards. A 0.025 M citrate buffer, pH 6.0, containing 0.2 M KCl, 0.1% Brij 35, and 0.02% NaN_3 was used.*

TABLE 1. *Purification of I_A*

Step	Fraction	Volume (ml)	Protein (mg/ml)	U/ml	U/mg	Recovery (%)	Purification
1.	Dialyzed 95 C extract	550	1.3	9.2	7.1	100	1
2.	DEAE-cellulose eluate	460	0.56	6.4	11.4	58	1.6
3.	Sephadex G-75 eluate	250	0.35	8.2	23.4	41	3.3
4.	DEAE-cellulose eluate	20	0.20	31	155	12	22

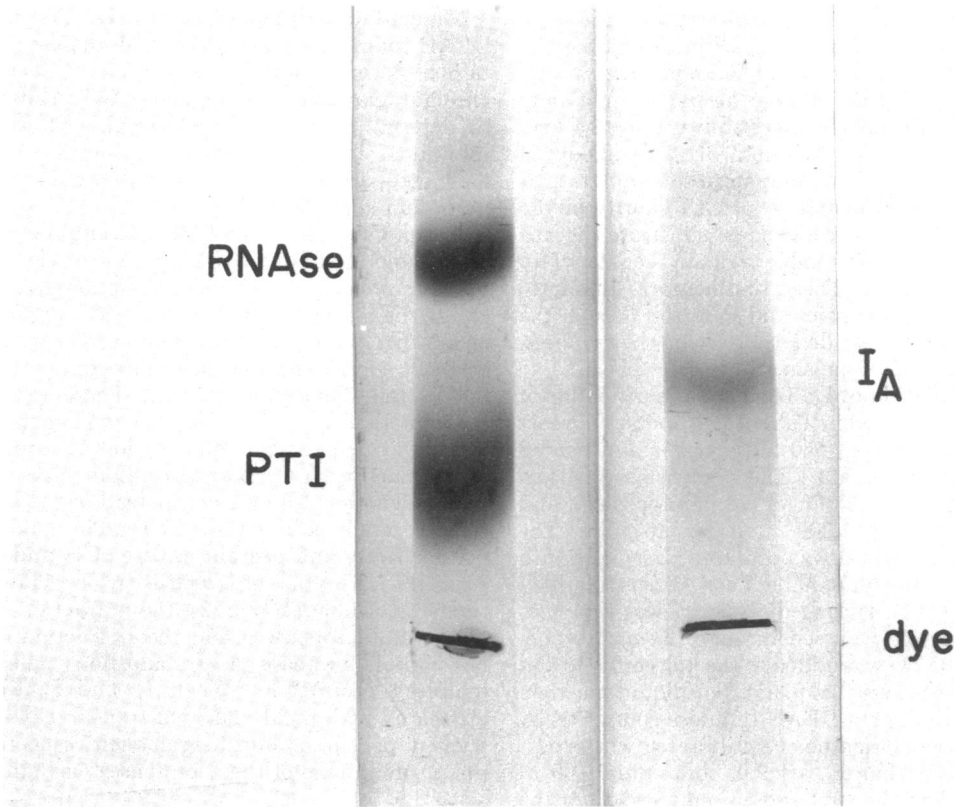

FIG. 3. *Sodium dodecyl sulfate-acrylamide gel electrophoresis of I_A. Bio-Rad precast 12% gels were used according to Weber et al. (33) and were stained with Coomassie brilliant blue R250. Sections of a duplicate unstained gel were macerated and extracted; when the extracts were analyzed, I_A activity was found only in the major band at molecular weight 11,000. Marker proteins used were pancreatic trypsin inhibitor (molecular weight 6,500), ribonuclease (molecular weight 13,700) and horse heart cytochome c (not shown, molecular weight 12,400).*

ited. In each case I_A and I_B were destroyed, indicating that they are protein molecules. In addition, pure protease C destroyed I_B at pH 5 and 7.

Effect of yeast proteases on I_A. Figure 4 illustrates that protease A did not digest I_A at pH 3 to 7. Protease B destroyed I_A most rapidly at pH 5 to 9, but was still quite effective at pH 3. Pure protease C (13) destroyed only 3 to 5% of I_A in 2 h at 30 C in its optimum pH range of 5 to 7. It appears that the activation of protease A which occurs in a yeast autolysate (18) could be attributable to the action to protease B, unless another protease such as aminopeptidase is also active in cleaving I_A.

Effect of pH on the stability of I_A and I_B. When inhibitor preparations were incubated for 16 h at 4 C, I_A and I_B were completely stable in the pH range of 3 to 10. At pH 2 and 11, I_A was stable, whereas I_B was somewhat unstable. The effect of protease B on the stability of I_B is

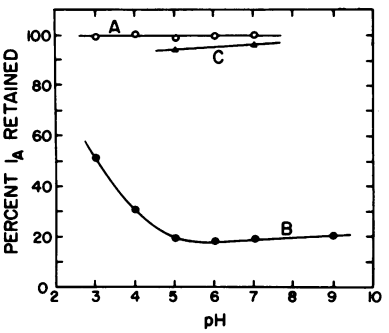

FIG. 4. *The effect of three yeast proteases on I_A. Protease A was a partially purified preparation containing 0.5 mM p-chloromercuribenzoate. Protease B was prepared by the procedure of Lenney and Dalbec (20) and contained no detectable A or C. Protease C (pure) was used at a level of 20 μg/ml. Incubation with I_A was at 30 C for 1 h (protease B) or 2 h (proteases A and C). Enzyme activity was destroyed by heating at 80 C for 10 min before analyzing for residual I_A.*

illustrated in Fig. 5. The protease B·I_B complex is stable at pH 6 to 10. Below pH 6 the complex appears to dissociate and I_B becomes a substrate instead of an inhibitor; pH 4.7 is the optimum pH for the digestion of I_B by protease B. At lower pH values, protease B is unstable and is less effective. Highly purified protease A (a DEAE-Sephadex eluate containing no protease B or C) rapidly destroyed I_B at pH 4.0.

Figure 6 illustrates the stability of I_A and I_B at 100 C. I_A was slightly more stable than I_B and also had a broader pH range of stability than I_B.

I_A and I_B calibration curves. The inhibition of protease A caused by increasing levels of I_A is illustrated in Fig. 7. The curve levels off at about 80% inhibition. When an excess of I_A was tested against highly purified protease A, the maximal attainable inhibition was 45% at pH 2.5, 84% at pH 3.5, and 100% at pH 4.5. Thus I_A seems to lose effectiveness as the pH is lowered.

The I_B calibration curve shown in Fig. 7 is linear up to 90% inhibition of protease B and then it approaches 100% inhibition. Tsai et al. (32) obtained an I_B calibration curve which was linear only up to 60% inhibition. It seems probable that their soluble casein substrate competes more effectively with I_B than does our insoluble azocoll substrate.

In the case of both of these inhibitors, complex formation was found to be complete in less than one minute. Partially inhibited proteases produced linear reaction kinetics, indicating that the enzyme-inhibitor complexes were not dissociating and the inhibitors were not being destroyed during the assay procedures. In both cases, one unit of inhibitor caused one unit of inhibition, irrespective of the level of protease A or B used in the assay.

Effects of actinomycete inhibitors on the yeast proteases. The four well known actinomycet protease inhibitors were tested against three yeast proteases with the results shown in Table 2. Pepstatin selectively inhibited protease A, whereas chymostatin and antipain selectively inhibited protease B. Leupeptin had no effect on proteases A, B, or C at the level tested.

Extraction and purification of the protease C·I_C complex at 25 C. Four pounds of yeast were plasmolyzed with chloroform as previously described (20). After 3 h at pH 7, the autolysate was centrifuged and the supernatant liquid was dialyzed with agitation overnight at 4 C against two changes of distilled water. The bag contents were applied to a DEAE-cellulose column (42 by 4 cm) which was equilibrated with 0.01 M *N*-ethylmorpholine buffer, pH 7.0. The protease B·I_B complex was eluted with the same buffer

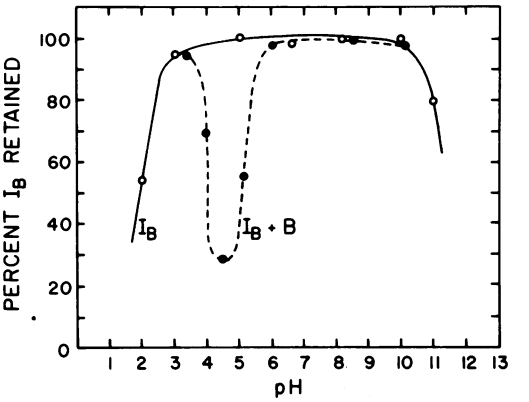

FIG. 5. *The effect of pH on the stability of I_B in the presence and the absence of protease B. Incubation was for 16 h at 4 C. Protease B and I_B were prepared as described by Lenney and Dalbec (20). The protease B preparation contained no detectable protease A or C. Protease B was destroyed by heating for 10 min at 80 C (pH 5 to 7) before analyzing for residual I_B.*

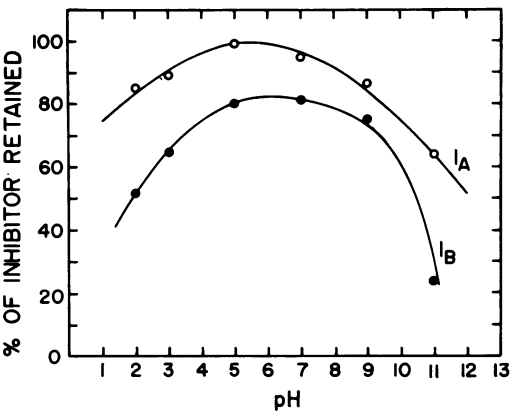

FIG. 6. *The effect of pH on the stability of I_A and I_B at 100 C. A DEAE-cellulose eluate (Table 1, step 2) containing both inhibitors was held at 100 C for 15 min. All aliquots were then adjusted to pH 5 to 7 and analyzed for residual inhibitors.*

containing 0.1 M NaCl. Then the protease C·I_C complex was eluted with this buffer containing 0.4 M NaCl. Ten milliliters of the protease C·I_C fraction was applied on a Sephadex G-100 column (44 by 2 cm) which was equilibrated with a 0.01 M phosphate buffer, pH 7.0, containing 0.01 M NaCl and 0.05% Brij. The protease C·I_C complex eluted from this column in some of the earlier protein fractions, followed by free I_C in some of the lower molecular weight protein fractions.

Partial separation of protease C and I_C. The protease C·I_C complex from the Sephadex column was applied to an DEAE-Sephadex

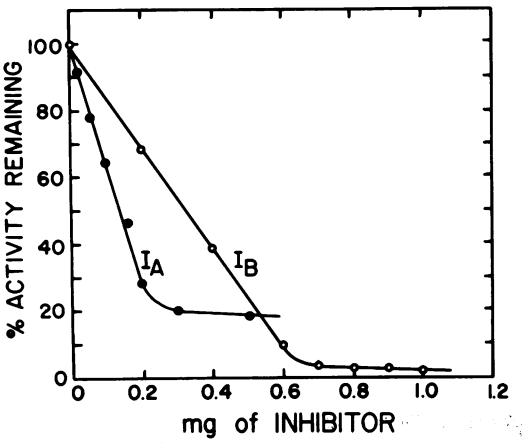

FIG. 7. *Calibration curves for I_A and I_B. The inhibitors were obtained from the two Sephadex G-75 eluate peaks depicted in Fig. 2. In separate experiments, increasing amounts of inhibitor protein were added to a constant level of our crude reference protease preparation.*

TABLE 2. *Effect of actinomycete inhibitors on yeast proteases*[a]

Inhibitor	Quantity (μg)	% inhibition of		
		Protease A	Protease B	Protease C
Pepstatin	0.5	100	0	0
Chymostatin	0.5	0	100	0
Leupeptin	1.0	0	0	0
Antipain	1.0	0	100	0

[a] Inhibitors were incubated with our reference enzyme preparation for 5 min at pH 6 before analyzing for residual protease A, B, or C. The inhibitor quantities represent the amounts present in the aliquots used in the assays.

A-50 column (8 by 1.5 cm) which had been equilibrated with 0.01 M phosphate, pH 7.0. When this column was eluted with 0.01 M phosphate containing 0.2 M NaCl, free I_C was found in the eluate. Further elution with this buffer containing 0.4 M NaCl produced an eluate containing a mixture of free C and protease C·I_C. This procedure was repeated using several other Sephadex G-100 eluates, and each time about one-third of the protease C·I_C complex decomposed, with the liberation of free protease C and I_C.

Some properties of I_C. Free I_C is an extremely labile molecule. It was necessary to work with it on the same day it was prepared because most of it was lost during storage at 4 C overnight, possibly because of destruction by contaminating proteases. I_C was destroyed by freezing and thawing; repeated freezing and thawing brought about the activation of protease C by destroying its inhibitor. Lyophilization of protease C·I_C destroys not only I_C but also about 90% of protease C itself. Hayashi et al. (12) have used solvents such as 30% dioxane or isopropanol as activators of the inactive form of this enzyme. However, in our hands these solvents produced erratic results, possibly because they caused some destruction of protease C as well as I_C. The most reliable procedure we found for the activation of the complex was overnight incubation at pH 4.6, under which conditions I_C was destroyed proteolytically. We were unable to find a means of treating protease C·I_C so as to selectively destroy protease C. Under all conditions tried, protease C was more stable than I_C.

Competitive inhibition of protease C by I_C. A sample of protease C·I_C was purified as described above by adsorption and elution from DEAE-cellulose and passage through Sephadex G-100. Several levels of this preparation were analyzed using Z-Glu-Tyr and ATEE as substrates, both before and after activation at pH 4.6. The resulting enzyme calibration curves are shown in Fig. 8. Protease C·I_C had considerable activity against Z-Glu-Tyr at pH 5, the shape of the calibration curve indicating that the complex was dissociating upon dilution (6). However, protease C·I_C had essentially no activity against ATEE at pH 8. Destruction of I_C produced large increases in both activities. This experiment indicates that the peptide substrate

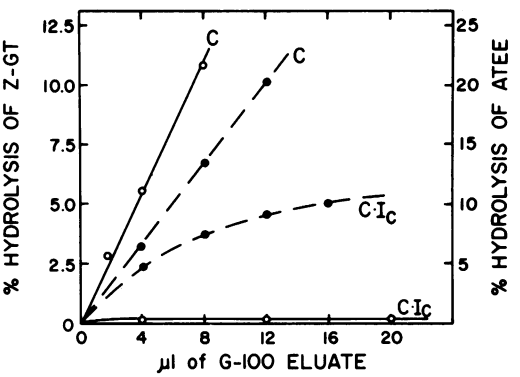

FIG. 8. *Action of several levels of protease C on ATEE and Z-Glu-Tyr before and after destruction of the accompanying I_C by overnight incubation at pH 4.6. The solid curves represent the hydrolysis of 0.01 M ATEE and the broken curves the hydrolysis of 2.5 mM Z-Glu-Tyr measured as described in the Materials and Methods section. The values shown along the abscissa refer to the Z-Glu-Tyr experiment; four times as much enzyme was used when ATEE was used as substrate.*

competes with I_C for protease C much more effectively than does the ester substrate. However, the pH of digestion should also be considered. At pH 5, the enzyme-inhibitor complex probably dissociates and some of I_C may be digested during the assay, whereas the complex is probably stable at pH 8.

I_C calibration curve. Although I_C may be attacked by protease C during the assay for I_C at pH 5.0, the calibration curve for I_C was linear and full inhibition of protease C was observed. The free I_C for this experiment was a Sephadex G-100 fraction which previously had been eluted from DEAE-cellulose as described above. Five levels of this fraction were analyzed against a constant amount of protease C. The results are shown in Fig. 9.

Free I_C in crude yeast autolysates. A sample of yeast was autolyzed for 48 h at pH 7 at 25 C as described above. At intervals, aliquots of the autolysate were clarified and were gel filtered to remove ninhydrin-positive material. Two-milliliter aliquots of clarified autolysate were passed through a Sephadex G-25 column (1.5 by 10 cm) by using a 0.01 M phosphate buffer containing 0.01 M NaCl and 0.05% Brij at pH 7.0. The break-through fractions were analyzed for I_C, and it was found that the level of free I_C increases rapidly between 18 and 24 h of autolysis, reaching a maximum at about 30 h. This observation indicates that yeast probably contains a surplus of I_C over the amount of protease C present. The presence of excess amounts of all three inhibitors apparently protects I_C from proteolytic destruction during the 30 h of autolysis.

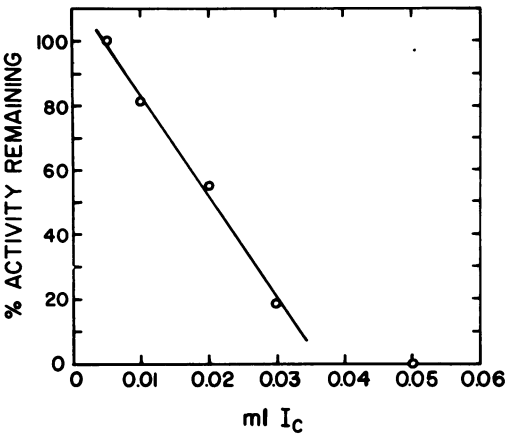

FIG. 9. *I_C calibration curve. Five levels of I_C were tested against a constant amount of protease C; 2.5 mM Z-Glu-Tyr at pH 5 was used as substrate for the assay of residual protease C.*

DISCUSSION

This is believed to be the first example of the presence in one cell of three inhibitors which specifically inhibit three proteases present in the same cell. The three proteases are localized in the vacuole in active form, whereas the inhibitors are present in excess in the extravacuolar cytosol (8, 21, 24). When the yeast cell is disrupted, the three enzyme-inhibitor complexes are formed. Six years ago the protease B·I_B complex was partially purified and I_B was found to be a thermostable protein with a molecular weight of 10,000 (20). In the present report, I_B was extracted as the free inhibitor; its properties appear to correspond to those of the inhibitor originally extracted as a complex.

Recently four research groups have studied the inhibitory effects of I_B on various proteolytic reactions. Cabib and Ulane (4) have examined the proteolytic activation of yeast chitin synthetase and the properties of an inhibitor of this reaction. Holzer et al. (15) have studied the inactivation of yeast tryptophan synthase by protease B and the inhibition of this reaction by I_B. Schott and Holzer (31) have obtained protease B in pure form and Hasilik and Holzer (9) have confirmed that protease B is capable of activating chitin synthetase. Tsai et al. (32) have studied the inactivation of yeast and *Neurospora* tryptophan synthase by a yeast protease (presumably protease B) and the inhibition of this destruction by an inhibitor (presumably I_B). Molano and Gancedo (25) have reported that a yeast protease inactivates yeast fructose 1,6-bisphosphatase and an inhibitor of this inactivation is also present in yeast. They concluded that protease B and its inhibitor were probably responsible for these effects. Hasilik and Holzer (8) showed that tryptophan synthase is stable in a suspension of lysed protoplasts as long as the vacuoles remain intact. Therefore, for the yeast proteases and their inhibitors to exert a regulatory role on extravacuolar enzymes, the proteases would have to leave the vacuole or the enzymes would have to enter the vacuole. Another possibility would be that the proteolysis might occur at the vacuolar membrane. Chen and Miller (5) have reported that protease A is detectable at the cell surface only during sporulation, indicating that this enzyme is not confined to the vacuole under these particular conditions. It is well known that the proteases in a suspension of disintegrated yeast cells will digest over 80% of the other proteins which are present. Therefore it is difficult to decide which in vitro activities of the proteases represent normal in vivo activities. However, the specificity of the three pro-

tease inhibitors suggests that they might be expected to play a role in regulating protein turnover in vivo.

Recently Betz et al. (3) obtained two molecular forms of I_B in highly purified condition. The two molecules differed only with respect to isoelectric point and appeared to be identical with the I_B previously characterized (20). Points of similarity include molecular weight, thermostability, specificity, digestibility by proteases A and B, speed of complex formation, and molecular weight of the complex. In both reports, inhibition was competitive when partially purified preparations were used. Laskowski and Sealock (17) have stated that all inhibitions by protease inhibitors are competitive, although the dissociation constants are extremely small in some cases.

The inhibitor of protease A bears a superficial resemblance to I_B. However the two molecules differ greatly in specificity and molecular weight, and differ slightly in thermostability. In addition, whereas proteases A, B, and C destroy I_B, only protease B has a pronounced destructive effect on I_A. While this paper was in preparation, Saheki et al. (29) described the purification and properties of I_A. They reported that this inhibitor is a tetramer which dissociates into subunits or monomers having a molecular weight of 6,100.

The observation that pepstatin is a potent inhibitor of protease A may be useful in preventing unwanted proteolysis when isolating proteins from yeast (see [28]). Diisopropylfluorophosphate, *p*-chloromercuribenzoate, or heavy metal ions may be used to inhibit the destructive action of proteases B and C.

The protease C·I_C complex was sufficiently stable to permit its partial purification by chromatography on Sephadex and DEAE-cellulose columns. However, the complex was partially dissociated during DEAE-Sephadex chromatography. The inhibition of protease C was competitive when a peptide substrate was used, but noncompetitive when ATEE was used as the substrate. Free I_C was very difficult to handle because of its extreme lability. Hayashi et al. (11, 12) isolated the inactive form of protease C and found that it consisted of the enzyme with a molecular weight of about 60,000 and a cationic subunit with a molecular weight of about 19,000. Their cationic subunit presumably corresponds to the present I_C. Activation by protein denaturants and by protease A probably represented the loss of the inhibitory activity of I_C. Very recently, I_C was obtained in pure form by Matern et al. (23).

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