Isolation and Properties of Two Inhibitors of Proteinase B from Yeast

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SUMMARY

In continuation of our studies on the inactivation of tryptophan synthase from Saccharomyces cerevisiae by proteinases from the same organism, two protein inhibitors of the tryptophan synthase inactivating yeast proteinase B were purified from boiled bakers’ yeast extract. The molecular weight of both inhibitors is 10,000 and both inhibit specifically proteinase B, but not proteinases A and C. The inhibitors form tight complexes with proteinase B. With the substrates Azocoll and N-acetyltyrosine ethyl ester, noncompetitive inhibition patterns are obtained.

The inhibitors are proteins with isoelectric points of 8.0 and 7.0, respectively. They are very stable in the pH range of 1 to 10, but they are easily destroyed by incubation with proteinase A and, less effectively, with proteinase B at pH 5.0.

A close molecular relationship of both inhibitors is indicated by the cross-reaction of inhibitor I with antiserum prepared against inhibitor II.

MATERIALS AND METHODS

Chemicals

Bovine serum albumin (from beef, dried, pure) was obtained from Behringwerke AG (Marburg a.d. Lahn, Germany); Azocoll from Calbiochem (Los Angeles, Calif.); Freund’s complete adjuvant from Difco Laboratories (Detroit, Mich.); kallikrein-trypsin inhibitor from BayerWerke (Elberfeld, Germany); trypsin, soybean trypsin inhibitor, RNase, cytochrome c, and hemoglobin were from Boehringer Mannheim GmbH (Mannheim, Germany); streptomycin sulfate was from “B. & K. O”-Bäckereinkauf E.G.m.b.H., Freiburg i. Br. The haploid wild type strain 2X180-1B was originally isolated by R. Mortimer (University of California, Berkeley).

Yeast Strains

Bakers’ yeast (Pleser Hefe, Darmstadt-Eberstadt) was obtained from “B&KO”-Bäckereinkauf E.G.m.b.H., Freiburg i. Br. The inhibitor was isolated and characterized by Katsunuma et al. (2) using yeast synthsas is activity and inhibition. The reaction mixture contained yeast inhibitor (inactivase Step 3, 17 μl of inhibitor fraction per ml of reaction mixture). After 15 min of incubation at 37° the reaction was stopped by the addition of 1 ml of 0.5 mM pyridoxal phosphate buffer, pH 7.0; 0.01 mM EDTA; 2.5 mM pyridoxal phosphate; 0.1 mM L-serine; and 0.8 mM indole. The reaction mixture contained yeast inhibitor (inactivation Step 3, 17 μl of inhibitor fraction per ml of reaction mixture). The amount of enzyme was determined by the factor 6.7 X 10³ to yield the micromoles of indole converted per min. One unit of tryptophan synthase is defined as the amount of enzyme that converts 1 μmole of indole to tryptophan in 1 min at 37°.

Assay of Enzyme Activities

Tryptophan Synthase Activity—Activity was measured by a modification of the method of Yanofsky (8) developed by Hasilik in our laboratory. This method takes advantage of a direct color reaction between the unconverted indole and pyridoxal phosphate in acid solution. Enzyme samples and controls without enzyme were made up to 70 μl with 0.1 mM potassium phosphate buffer, pH 7.0. The reaction was started by the addition of 100 μl of freshly prepared reaction mixture (0.1 mM potassium phosphate buffer, pH 7.0; 0.01 mM EDTA; 2.5 mM pyridoxal phosphate; 0.1 mM L-serine; and 0.8 mM indole). The reaction mixture contained yeast inhibitor (inactivation Step 3, 17 μl of inhibitor fraction per ml of reaction mixture). After 15 min of incubation at 37° the reaction was stopped by the addition of 1 ml of 0.5 mM FeCl₃ in 8% HCl, and the samples were incubated for color development at 95° for 10 min. Precipitated protein was removed by centrifugation for 3 min at 8,000 X g, and the absorbancies of the samples were read at 546 μm. The sample absorbance was subtracted from the control value without enzyme. The resulting extinction difference, which corresponded to the amount of indole converted, was multiplied by the factor 6.7 X 10³ to yield the micromoles of indole converted per min. One unit of tryptophan synthase is defined as the amount of enzyme that converts 1 μmole of indole to tryptophan in 1 min at 37°.

Tryptophan Synthase Inactivating Activity—All inactivation reactions were measured according to Katsunuma et al. (2) using

1. A. Hasilik, manuscript in preparation.
the above described method of Hasilik; incubations of tryptophan synthase with inactivating enzymes in a total volume of 70 µl were usually for 20 min. Units of inactivating activity were calculated using zero order interpolation as described by Saheki and Holzer (4).

Protease A—Protease A was assayed with acid-denatured hemoglobin according to Hata et al. (9) as modified by Saheki and Holzer (4). Specific activity is given as units per mg of protein.

Protease B—Protease B was routinely assayed with Azocoll according to Saheki and Holzer (4). Specific activity is given as units per mg of protein. In a few cases the N-acetyl-L-tyrosine ethyl ester esterolytic activity of this enzyme was measured by the pH-stat method of Hata et al. (9) in a total volume of 2.5 ml at a final concentration of 10 mM using the pH-stat assembly ABU 12/TTT 11/PHM26/SBR2/TT31 (Radiometer, Copenhagen, Denmark).

Protease C—Hydrolysis of N-acetyl-L-tyrosine ethyl ester was measured by the pH-stat method of Hata et al. (9) as described for protease B.

Assay of Inhibitory Activity

Inhibitory activity was assayed with the above described methods either against a preparation of tryptophan synthase inactivating enzymes or against protease B. Generally, inhibitory activity was expressed as the amount of enzyme activity inhibited per ml of inhibitor solution.

\[
\text{Inhibitor activity} = \frac{x - y}{\text{ml of inhibitor solution}}
\]

where \(x\) = proteinase activity without inhibitor and \(y\) = proteinase activity with inhibitor. For \(y\), inhibition values between 30 and 60\% inhibition were used for calculation.

Usually enzyme and inhibitor were combined at least 1 min before addition of the substrate. Only when \(N\)-acetyl-L-tyrosine ethyl ester was used as a substrate for protease B was the inhibitor added to the proteinase-substrate mixture. In this case, the free reduction of the rate of esterolysis by the inhibitor was observed immediately after the addition of the inhibitor (20 s or less).

Protein was estimated by the method of Lowry et al. (10) using crystalline bovine serum albumin as a standard.

Disc gel electrophoresis was carried out in the Buchler Poly-analyst apparatus. The anodic gel system described in Buchler's instruction manual was used. Gels were usually stained with Coomassie brilliant blue, as described by Mason et al. (11). Where indicated, gels were divided into 2-mm slices with an egg slicer.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to the method of Mason et al. (11) in 16\% polyacrylamide gels.

For isoelectric focusing in 7.5\% polyacrylamide gels, the method of Wrigley (12) was followed. Carrier ampholytes were removed by extensive washing of the gels in 5\% trichloroacetic acid. The gels were stained with 0.5\% amido black in 7.5\% acetic acid.

Preparation of Antisera to Yeast Inhibitor II—Antiserum was prepared in rabbits as described by Mason et al. (11). Control serum was taken from the rabbits before injection of the antigen.

Each rabbit received 1 mg of inhibitor II protein in 1 ml of 0.1 M potassium phosphate buffer, pH 7.0, and then 1.5 ml of Freund's complete adjuvant. The antigen was administered in multiple subcutaneous injections on the back and into two footpads. After 4 weeks, each animal received a booster injection of 1 mg of inhibitor II into the ear vein. On the 6th, 8th and 10th days after the booster injection, blood was collected from the ear vein and allowed to clot overnight in the refrigerator. After centrifugation at 5000 X g for 10 min, the resulting serum was stored at -20°.

Immunoprecipitation was studied by the double diffusion technique of Ouchterlony (13) in 0.8\% agar plates containing 0.14 M NaCl, 0.047 M Tris-HCl, pH 7.7, and 0.006 M methionolate.

Purification of Enzymes

If not otherwise stated, all steps were carried out at 4°. The enzyme preparations were stored at -20°.

Tryptophan Synthase—This was purified about 10-fold from \(S.\) \(cerevisiae\) X2180-1B by following the procedure described by Katsunuma et al. (2) up to and including Step 4. Step 4 was modified by fractional salting out from 0 to 150 g of ammonium sulfate per liter first, and then from 150 to 250 g of ammonium sulfate per liter. Usually the second fraction contained the higher specific activity.

Tryptophan Synthase Inactivating Enzymes—A preparation of tryptophan synthase inactivating enzymes according to Step 5 of the purification procedure of Katsunuma et al. (2) was used for the assay of tryptophan synthase inactivating enzymes inhibitory activity. In some experiments purified proteases A and B as described below were used as inactivating enzymes.

Purification of Yeast Proteases—The purification of yeast proteases was carried out similarly to the methods of Hata et al. (9). Two kilograms of bakers' yeast in 2 liters of 0.1 M potassium phosphate buffer, pH 7.0, were homogenized by passing the suspension five times through a Manton-Gaulin homogenizer (Manton-Gaulin Mfg. Co., Oskaloosa, Mass.) at 5,000 g per cm². The effluent was cooled to 0°. The homogenate was centrifuged at 27,000 X g for 30 min, and the pH of the resulting crude extract (supernatant 2,500 ml) was adjusted to pH 5.0 with 20\% acetic acid. Then 100 mg of penicillin G (potassium salt) and 100 mg of streptomycin sulfate were added, and the extract was incubated at 25°. After 20 hours, ammonium sulfate was added to 50\% saturation; the suspension was stirred for 2 hours and then centrifuged at 27,000 X g for 30 min. The precipitate was dissolved in 0.01 M potassium phosphate buffer, pH 7.0, and extensively dialyzed against the same buffer. After centrifugation with 27,000 X g for 30 min, the resulting solution (800 ml) was applied to a DEAE-Sephadex A-50 column (5 X 33 cm), equilibrated with 0.01 M potassium phosphate buffer, pH 7.0, at a flow rate of 10 ml per hour. After washing the column with 1 liter of 0.01 M potassium phosphate buffer, pH 7.0, a linear gradient from 0 to 0.4 M NaCl in the same buffer was applied to the column (total volume 3 liters; 6 fractions per hour). The fractions with proteinase B and protease A activity were pooled separately and then dialyzed against 10 liters of 0.01 M potassium phosphate buffer, pH 7.0, overnight. DEAE-Sephadex A-50, 200 ml (swollen in 0.01 M potassium phosphate buffer, pH 7.0) was added to each dialyzed protease sample, and, after 1 hour of stirring, the DEAE-Sephadex was poured into a column of 2.5-cm diameter. Proteinase A was eluted from the column with a linear gradient from 0 to 0.4 M NaCl. Proteinase B with a linear gradient from 0 to 0.3 M NaCl, both in 0.01 M potassium phosphate buffer, pH 7.0, a linear gradient from 0.01 M potassium phosphate buffer, pH 7.0, a linear gradient from 0 to 0.4 M NaCl in the same buffer was applied to the column (total volume 3 liters; 6 fractions per hour). The fractions with proteinase B and proteinase A activity were pooled separately and then dialyzed against 10 liters of 0.01 M potassium phosphate buffer, pH 7.0, overnight. DEAE-Sephadex A-50, 200 ml (swollen in 0.01 M potassium phosphate buffer, pH 7.0) was added to each dialyzed protease sample, and, after 1 hour of stirring, the DEAE-Sephadex was poured into a column of 2.5-cm diameter. Proteinase A was eluted from the column with a linear gradient from 0 to 0.4 M NaCl, and proteinase B with a linear gradient from 0 to 0.3 M NaCl, both in 0.01 M potassium phosphate buffer, pH 7.0, and then centrifuged at 27,000 X g for 30 min. The precipitate was dissolved in 0.01 M potassium phosphate buffer, pH 7.0, and stored in 0.1-ml portions.

The specific activity of the proteinase A preparation obtained by this procedure was 770 units per mg of protein; the contaminating protease B activity in this preparation was 0.085 A₅₂₀ per mg of protein. The content of proteinase A activity was measured by the method of Mason et al. (9) as described below were used as inactivating enzymes.

Preparation of Antisera to Yeast Inhibitor II—Antiserum was prepared in rabbits as described by Mason et al. (11). Control serum was taken from the rabbits before injection of the antigen. Each rabbit received 1 mg of inhibitor II protein in 1 ml of 0.1 M potassium phosphate buffer, pH 7.0, and then 1.5 ml of Freund's complete adjuvant. The antigen was administered in multiple subcutaneous injections on the back and into two footpads. After 4 weeks, each animal received a booster injection of 1 mg of inhibitor II into the ear vein. On the 6th, 8th and 10th days after the booster injection, blood was collected from the ear vein and allowed to clot overnight in the refrigerator. After centrifugation at 5000 X g for 10 min, the resulting serum was stored at -20°.

Immunoprecipitation was studied by the double diffusion technique of Ouchterlony (13) in 0.8% agar plates containing 0.14 M NaCl, 0.047 M Tris-HCl, pH 7.7, and 0.006 M methionolate.

Tryptophan Synthase—This was purified about 10-fold from \(S.\) \(cerevisiae\) X2180-1B by following the procedure described by
TABLE I

Purification of yeast inhibitors

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Inhibition of tryptophan synthase inactivase activity</th>
<th>Inhibition of proteinase B (substrate Azocoll)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total activity</td>
<td>Specific activity</td>
</tr>
<tr>
<td></td>
<td>ml</td>
<td>millions</td>
<td>millions/mg protein</td>
</tr>
<tr>
<td>1.</td>
<td>5,000</td>
<td>27,000</td>
<td>1.02</td>
</tr>
<tr>
<td>2.</td>
<td>215</td>
<td>15,250</td>
<td>1.73</td>
</tr>
<tr>
<td>3.</td>
<td>54</td>
<td>9,500</td>
<td>3.31</td>
</tr>
<tr>
<td>4.</td>
<td>6.2</td>
<td>4,055</td>
<td>16.0</td>
</tr>
<tr>
<td>5.</td>
<td>20.8</td>
<td>2,140</td>
<td>43.0</td>
</tr>
<tr>
<td>6.</td>
<td>2.5</td>
<td>294.1</td>
<td>7.0</td>
</tr>
<tr>
<td>7A.</td>
<td>4.3</td>
<td>208.2</td>
<td>60.3</td>
</tr>
<tr>
<td>7B.</td>
<td>1.2</td>
<td>31.3</td>
<td>68.7</td>
</tr>
<tr>
<td>8.</td>
<td>3.1</td>
<td>176.9</td>
<td>136.3</td>
</tr>
</tbody>
</table>

* Pooled peaks I from Step 6 set to 100%.

Step 1: Preparation of Crude Boiled Yeast Extract

Bakers' yeast (5 kg) was suspended in 5 liters of 0.1 M potassium phosphate buffer, pH 7.0. Portions of this suspension (600 ml) were heated in a boiling water bath for 20 min and then immediately cooled in ice. Centrifugation at 27,000 × g_{max} for 15 min yielded a yellow, lipid-containing supernatant.

Step 2: Precipitation by Trichloroacetic Acid

The boiled yeast extract was brought to 15% saturation (w/v) with 100% trichloroacetic acid and stirred gently overnight. The suspension was then centrifuged at 27,000 × g_{max} for 30 min. The precipitate was dissolved in 0.1 M potassium phosphate buffer, pH 7.0, the pH being adjusted to 7.0 with 2 N KOH.

Step 3: Ethanol Fractionation

The resulting solution was cooled in an ice bath containing enough salt to prevent a temperature rise above 2°C. Absolute ethanol stored at -20°C was added to 45% (v/v) final concentration. After being stirred for 20 min at 4°C, the solution was centrifuged for 10 min at 27,000 × g_{max} for 30 min. The precipitate was dissolved in 0.1 M potassium phosphate buffer, pH 7.0, and dialyzed against three changes of 0.01 M potassium phosphate buffer, pH 7.0, and dialyzed against three changes of water.

Step 4: DEAE-Sephadex Chromatography

The dialyzed ethanol fraction was applied to a DEAE-Sephadex A-50 column (2.5 × 40 cm) equilibrated with 0.01 M potassium phosphate buffer, pH 7.0. The column was slushed with the same buffer at a flow rate of 30 ml per hour; fractions were changed every 20 min. Inhibitory activity was found mainly at the second part of the protein peak. The active fractions were concentrated by freeze drying to approximately 5 ml.

Step 5: Sephadex G-75 Chromatography

The concentrated DEAE-Sephadex eluate was applied to a Sephadex G-75 column (2.5 × 120 cm) equilibrated with 0.01 M potassium phosphate buffer, pH 7.0. The flow rate was 30 ml per hour; fractions were collected at 10-min intervals. The active fractions within the bulk of the main protein peak were pooled and freeze-dried.

Step 6: QAE-Sephadex Chromatography (Separation of Inhibitors I and II)

The dry material from Sephadex G-75 was dissolved in water and dialyzed against 0.01 M Tris-HCl buffer, pH 9.0. The dialyzed solution was applied to a QAE-Sephadex A-50 column (2.0 × 25 cm), equilibrated with the Tris-HCl buffer. The column was washed with about 150 ml of the same buffer at a flow rate of 30 ml per hour; fractions were changed every 10 min. Then a linear gradient from 0 to 0.2 M NaCl in 0.01 M Tris-HCl buffer, pH 9.0, was applied (total volume 500 ml). Inhibitor I was eluted with the main protein peak, whereas inhibitor II was found in the following fractions (Fig. 1). The corresponding fractions were pooled and freeze-dried. The dry material was dissolved in 0.01 M potassium phosphate buffer, pH 7.0.

Step 7: Final Purification of Inhibitors

A: Inhibitor I—For purification of inhibitor I, usually the material of two or three purification procedures was pooled. The
material was dialyzed against 0.05 M sodium acetate buffer, pH 5.5, and applied to an SP-Sephadex C-25 column (20 x 20 cm), equilibrated with the same buffer at a flow rate of 30 ml per hour; fractions were changed every 10 min. The column was washed with about 150 ml of the buffer. Then a linear gradient from 0 to 0.25 M NaCl in 0.05 M sodium acetate, pH 5.5, was applied (total volume 500 ml). Inhibitory activity was eluted at approximately 0.15 M NaCl concentration, and was clearly separated from other, earlier appearing protein peaks. The active fractions were pooled, freeze-dried, dissolved in water, and dialyzed against 0.1 M potassium phosphate buffer, pH 7.0.

**B: Inhibitor II** — Inhibitor II from the QAE-Sephadex column was found to be almost homogeneous by the criterion of disc electrophoresis. Complete purification could be obtained by either repeated chromatography of the material on QAE-Sephadex under the same conditions as described in Step 6, or by the same procedure as used for the purification of inhibitor I in Step 7A. In the latter case, the inhibitor was eluted from the SP-Sephadex column at approximately 0.1 M NaCl concentration. The active fractions were treated as described under Step 7A.

As shown in Table I, inhibitor I is purified about 140-fold and inhibitor II about 70-fold from boiled yeast extract as calculated from the inhibitory activities. The final yield of both inhibitors was usually between 0.1 and 0.5%. These values may not represent the true purification values since boiled yeast extract may contain other unspecific inhibitory material apart from the inhibitors I and II. This idea is supported by the different modes of inhibition obtained with boiled yeast extract and the purified inhibitors, which is discussed later.

**Properties of Inhibitors**

Both inhibitor preparations behaved homogeneously in disc electrophoresis (Fig. 2). Inhibitory activity in 2-mm slices from unstained gels coincided with the protein band in stained gels.

![Fig. 2. Disc electrophoresis of crude boiled yeast extract and purified inhibitors I and II. Disc electrophoresis was carried out in gels containing 7.5% (w/v) acrylamide, 0.2% (w/v) bisacrylamide, 0.06% (v/v) tetramethylethylenediamine, and 0.07% (w/v) ammonium persulfate in 0.37 M Tris-HCl, pH 8.9. The upper buffer contained 5.36 g of Tris and 3.48 g of glycine per liter, the lower buffer 14.5 g of Tris and 60 ml of 1 N HCl per liter. The gels (0.5 x 6.1 cm) were run at 20° with a current of 2.5 ma per tube, until the tracking dye, bromophenol blue (2 x 10^-4% (w/v) in the upper buffer), reached the bottom of the tube. The protein samples (1, 240 pg of crude boiled yeast extract; 2, 43 pg of inhibitor I; and 3, 40 pg of inhibitor II) were sliced into 2-mm slices; each gel slice was homogenized in 0.4 ml of 0.1 M potassium phosphate buffer, pH 7.0. Inhibitory activity was assayed with the Azocoll method.](http://www.jbc.org/)

Isoelectric focusing of the inhibitors in 7.5% polyacrylamide gels yielded single bands for both inhibitors. The estimation of pH in 2-mm slices from unstained reference gels showed that inhibitor I has an isoelectric point of approximately 8.0, inhibitor II of 7.0 (Fig. 3).

The molecular weights of the inhibitors were determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and by Sephadex G-75 gel filtration. The two inhibitors banded at the same position in 16% sodium dodecyl sulfate polyacrylamide gels. From calculation of the mobilities of the inhibitors and various reference proteins, the molecular weight of both inhibitors was calculated to be 10,000 _±_ 1,500 (Fig. 4). The molecular weights of the inhibitors as determined by assaying activity with Azocoll on a calibrated Sephadex G-75 column were both found to be 10,000 _±_ 1,000 and were therefore in agreement with the values determined by gel electrophoresis (Fig. 5).

The ultraviolet absorption spectra of both inhibitors at pH 7.0 were very similar and showed an absorption maximum at 277 nm. The A_{277}:A_{315} ratio for inhibitor I was found to be 1.27, for inhibitor II, 1.23.

The activities of both the crude inhibitor (Step 3) and of the purified inhibitors were found to be very stable in acid, neutral, and slightly alkaline media, when incubated at 37° for 5 hours (Table II).

**Interactions between Inhibitors and Proteinases**

The inhibitory activity of both inhibitors against purified proteinases A and B was assayed in the same test system with the tryptophan synthase inactivation reaction at pH 7.0 (Fig. 6). Both purified inhibitors inhibited proteinase B very effectively, whereas proteinase A was almost not inhibited. The N-acetyl-tyrosine ethyl ester esterolytic activity of proteinase C at pH 8.0 was not affected even by large amounts of partially purified inhibitor (Fig. 7).
The amount of proteinase B inhibition was constant between pH 6.0 and 9.0, whereas at pH 5.0 the inhibitory activity was decreased, probably because of a weaker association between proteinase and inhibitor in the acid pH range (Fig. 8).

Experiments with a calibrated Sephadex G-75 column showed that the inhibitory activities of both inhibitors, which normally were eluted with a molecular weight of 10,000, were shifted completely to a molecular weight of 44,000 after 15 min of incubation with proteinase B at 25° and pH 7.0 (Fig. 5). This indicates that a tight complex is formed between the proteinase and the inhibitors.

The high affinity of the inhibitors for proteinase B was further

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**TABLE II**

| pH stability of crude inhibitor (Step 3) and purified inhibitors I and II |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Remaining activity at pH    | %                           | %                           | %                           | %                           | %                           |
| Fraction                    | 1                           | 3                           | 5                           | 7                           | 9                           | 11                          | 13                          |
| Crude inhibitor (Step 3)    | 78                          | 88                          | 87                          | 94                          | 73                          | 89                          | 10                          |
| Inhibitor I                 | 86                          | 100                         | 81                          | 90                          | 110                         | 99                          | 21                          |
| Inhibitor II                | 74                          | 68                          | 76                          | 89                          | 88                          | 90                          | 36                          |

Fig. 4 (left). Molecular weight determination of inhibitor I and inhibitor II, proteinase B, and proteinase B-inhibitor complexes by Sephadex gel filtration. A Sephadex G-75 column (1.0 X 100 cm) was used throughout the experiments. The column was run with 7 ml of 0.01 M potassium phosphate buffer, pH 7.0, per hour; the fractions were collected every 8 min. V4.3, was estimated with blue dextran; calibration was obtained with 1 mg of cytochrome c and 2 mg of all other proteins.

Fig. 5 (right). Molecular weight determination of inhibitors I and II, proteinase B, and proteinase B-inhibitor complexes by Sephadex gel filtration. A Sephadex G-75 column (1.0 × 100 cm) was used throughout the experiments. The column was run with 7 ml of 0.01 M potassium phosphate buffer, pH 7.0, per hour; the fractions were collected every 8 min. V4.3, was estimated with blue dextran; calibration was obtained with 1 mg of cytochrome c and 2 mg of all other proteins.

Fig. 6 (left). Effect of inhibitors I and II on the thryptophan synthase inactivating activities of proteinases A and B. O - - - O, inhibitor I; ● - - - ●, inhibitor II. Tryptophan synthase inactivating activity was measured at pH 7.0 and 37° as described under "Materials and Methods." An equal amount of tryptophan synthase (1.0 mg of protein) and inhibitors (100 µg of protein each) were determined by the Azocoll method. Proteinase A (2.3 mg of protein) and inhibitors (100 µg of protein each) were determined by the Azocoll method.
FIG. 9. Lineweaver-Burk plots of the rate of hydrolysis of azocoll by proteinase B with crude boiled yeast extract and purified inhibitors I and II. Azocoll hydrolysis was measured according to Saheki and Holser (4). Inhibitors added: 12.5 pg (i) and 25 pg (ii) crude boiled yeast extract (Step 1) in A; 0.13 pg (i) and 0.26 pg (ii) inhibitor I in B; 0.2 pg (i) and 0.4 pg (ii) inhibitor II in C. Shown by kinetic experiments. The mode of inhibition of the two purified inhibitors and of crude boiled yeast extract was assayed using the Azocoll-hydrolyzing activity of proteinase B (Fig. 9). Crude boiled yeast extract inhibits proteinase B competitively, whereas the purified inhibitors inhibit noncompetitively. The use of the insoluble macromolecular substrate Azocoll for the determination of the mode of inhibition seems justified, since with N-acetyltyrosine ethyl ester as substrate for proteinase B, the same noncompetitive inhibition pattern for purified inhibitor II was obtained (experiment not shown).

As shown in Fig. 10, inhibitory activity is effectively destroyed by proteinase A and more slowly by proteinase B during incubation at pH 5.0 and 25°. In this experiment the amount of proteinase B was chosen so as to be inhibited to 90% and 45%, respectively. The amount of proteinase A added had about the same tryptophan synthase inactivating activity and protein content. The experiment shown was done with a mixture of both inhibitors (Step 5), but quite similar results were obtained with purified inhibitor II.

Immunological Cross-Reactivity of Inhibitor I and Inhibitor II

Data are shown in Fig. 11. The antiserum prepared against purified yeast inhibitor II showed one single, identical precipitation line with the Ouchterlony double diffusion technique against crude yeast extract, crude boiled yeast extract, ethanol precipitate (Step 3), Sephadex G-75 eluate (Step 5), and both purified inhibitors I and II. With control serum, no precipitation was observed.

DISCUSSION

The purification and some properties of two heat-stable inhibitors of proteinase B have been described. These two inhibitors were shown to have a molecular weight of 10,000, isoelectric points of 8.0 and 7.0, respectively, and to be very stable in the pH range from 1 to 10. Both inhibitors act noncompetitively on proteinase B, in contrast to the inhibitor of proteinase B reported by Lenney and Dalbec (14) which inhibits competitively. They form tight complexes with proteinase B, and they have little or no effect on the activity of proteinase A and proteinase C.

Since both inhibitors are destroyed by proteinase A and much more slowly by proteinase B, we assume that the activation of
proteinase B in autolysates from bakers' yeast reported by Hayashi et al. (15) is mainly due to a digestion of the inhibitors by proteinase A.

It seems possible that inhibitor II is a modification product of inhibitor I, first, since the specific activity of inhibitor I against proteinase B is higher than that of inhibitor II and, second, since both inhibitors react with antisera prepared against inhibitor II. This idea is supported further by the observation that the distribution of inhibitors I and II on QAE-Sephadex chromatography was not always constant: in some preparations only very little inhibitor I activity was observed with a concomitant increase of the peak of inhibitor II activity. The modification of inhibitor I may be connected with proteolytic alterations in the course of proteinase B activation. For example, splitting off one or a few basic amino acids from inhibitor I may explain the more acid isoelectric point of inhibitor II and may also be responsible for the differences in specific activity. A detailed investigation of the amino acid composition and the molecular structure of the inhibitors is in progress in our laboratory.

A possible physiological role of the tryptophan synthase inactivating, i.e., proteolyzing, enzyme for the regulation of enzyme activities and protein turnover has been discussed earlier (2, 7, 16). The function of the inhibitors within the regulation of the proteinase activity in yeast is at present poorly understood. Quite generally, the existence of specific inhibitors may open new possibilities for regulatory mechanisms within the field of controlled specific proteinase action. As shown by Hasilik and Holzer (17), a partially purified preparation of the inhibitors described in this paper, as well as pure inhibitor II, inhibited the proteinase B-catalyzed activation of prechitin synthase by limited proteolysis reported by Cabib and Ulane (18). Therefore, one of our inhibitors might be identical with, or closely related to, the inhibitor of the chitin synthase activating factor (19). It might be that the inhibitors also, in other cases, participate in the control of activation, inactivation, or modification of enzymes by limited proteolysis.

For control of protein turnover, it seems reasonable that yeast synthesizes inhibitors against its neutral proteinase B to prevent unnecessary degradation of its cellular proteins when enough amino acids are available. Since proteinase B is compartmentalized in a special organelle, the yeast vacuole (20–22), the inhibitors may act as safety devices within the cytoplasm. With regard to phylogeny they might be understood as evolutionary intermediates between a simple intracellular neutral preprenotease, which is only temporarily activated, and the specialized uninhibited degradation machinery in the mammalian lysosome, which works permanently at acidic pH. For instance, the peptide split off during activation of the preprenotease molecule could have obtained a new function as a proteinase inhibitor, allowing a better control of the proteinase activity by different compartmentalization. Preliminary evidence for such a different localization of proteinase B and the inhibitors was obtained in our laboratory.7,1

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