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Isolation and Partial Characterization of an Acid Carboxypeptidase from Yeast†

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ABSTRACT: The purification and characterization of a carboxypeptidase from baker's yeast, *Saccharomyces cerevisiae,* are described. The purified enzyme has been characterized with respect to molecular weight (62,000), amino acid composition, amino terminal sequence, enzymatic properties, and sensitivity to group-specific inhibitors. The enzyme is a glycoprotein composed of a single polypeptide chain, which contains 13 half-cystine residues, 2.6% amino sugars, and 12.7% neutral hexoses. The enzyme releases a variety of amino acids, including proline, from the carboxyl terminus of proteins. Unlike pancreatic carboxypeptidases, the yeast enzyme is not inhibited by chelating agents but is inhibited by diisopropyl phosphorofluoridate, *p*-mercuribenzoate, mercuric chloride, *N*-tosyl-L-phenylalanine chloromethyl ketone, phenylglyoxal, iodoacetamide, and by photooxidation in the presence of rose bengal. The enzyme retains full activity in 4 M urea and is useful for determining carboxyl-terminal sequences of proteins and peptides.

Two distinct classes of carboxypeptidases have been described. One class, typified by the pancreatic carboxypeptidases, exhibits a neutral to alkaline pH optimum and requires for activity divalent cations of the IIB transition series (Vallee and Riordan, 1969; Petra, 1970). The second class, termed "acid carboxypeptidases" (Zuber and Matile, 1968), displays maximal activity in the acid range and is inhibited by Dip-F.[1] In contrast to the metallocarboxypeptidases, acid carboxypeptidases release from the carboxyl terminus of proteins the imino acid proline, in addition to several other amino acids. Acid carboxypeptidases have been isolated from yeast (Hata *et al.,* 1967a,b), citrus peel (Zuber, 1964), citrus leaves (Zuber and Matile, 1968; Sprossler *et al.,* 1971; Tschesche and Kupfer, 1972), Aspergillus (Ichishima, 1972), bean leaves (Wells, 1965), cotyledons of germinating cotton seedlings (Ihle and Dure, 1972a,b), germinating barley (Visuri *et al.,* 1969), and have been observed in a variety of plant tissues (Zuber and Matile, 1968). Cathepsin A from bovine spleen also displays several characteristics of acid carboxypeptidases (Logunov and Orekhovich, 1972).

In contrast to the extensively studied metallocarboxypeptidases, relatively little is known about the structure and mechanism of action of the acid carboxypeptidases. In order to extend our knowledge of these enzymes, we have isolated an acid carboxypeptidase from baker's yeast. This enzyme was first described by Hata *et al.* (1967a) under the name of "protease C" and certain of its chemical and enzymatic properties have been reported (Hata *et al.,* 1967b; Aibara *et al.,* 1971; Hayashi *et al.,* 1970). The present paper describes a method of isolation of the enzyme and its characterization. While this work was in progress, Hayashi *et al.* (1973) described the isolation and some characteristic properties of this enzyme which are in close agreement with those reported herein.

Experimental Section

Materials

Compressed baker's yeast was obtained through the courtesy of Standard Brands, Inc.

N-Acetyl-L-tyrosine ethyl ester (Ac-Tyr-OEt) was purchased from Fox Chemical Co. All carbobenzoxy dipeptides were obtained from Cyclo Chemical Corp. The B-chain of oxidized bovine insulin (lot W-1300) was from Schwarz/Mann.

DEAE-cellulose was purchased from Schleicher and Schuell, Inc. and DEAE-Sephadex A-50 and Sephadex G-150 from Pharmacia Fine Chemicals.

The protein standards used for sodium dodecyl sulfate gel electrophoresis were all obtained from Worthington Biochemical Corp. except phosphorylase *b* which was a gift from Dr. Edmond H. Fischer.

Dip-F was purchased from Pierce Chemical Co. The reagent was diluted to 1 M in water-free 2-propanol and stored over molecular sieves (Matheson Coleman and Bell).

Tetranitromethane, *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide, and Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid)) were obtained from Aldrich Chemical Co., Inc. *N*-

† From the Department of Biochemistry, University of Washington, Seattle, Washington 98195. *Received April 25, 1974.* Taken in part from a dissertation submitted by R. W. Kuhn to the Graduate School of the University of Washington in partial fulfillment of the requirements for the degree of Doctor of Philosophy. A preliminary report has been published (Kuhn *et al.,* 1972). This work was supported by research grants from the National Institutes of Health (GM 15731) and the American Cancer Society (BC-91). R. W. K. was supported by a training grant from the National Institutes of Health (GM 00053) to the Department of Biochemistry.

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[1] Abbreviations used are: Dip-F, diisopropyl phosphorofluoridate; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; Ac-Tyr-OEt, *N*-acetyl-L-tyrosine ethyl ester; Cbz-Phe-Leu, benzyloxycarboxylphenylalanylleucine; Cbz-Gly-Leu, benzyloxycarboxylglycylleucine, $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride.

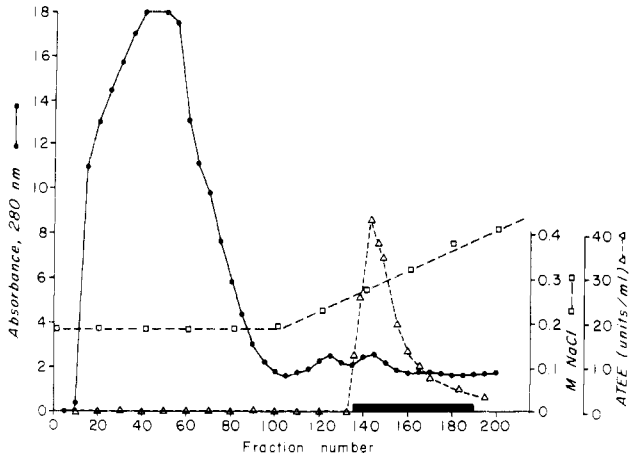

FIGURE 1: Chromatography on DEAE-cellulose of the 0–90% ammonium sulfate fraction of a yeast autolysate. The column (4.0 × 60 cm) was developed at a flow rate of 200 ml/hr and 20-ml fractions were collected. Units of activity are micromoles of substrate hydrolyzed per min per ml of solution. Fractions indicated by the bar were pooled for subsequent chromatography on DEAE-Sephadex.

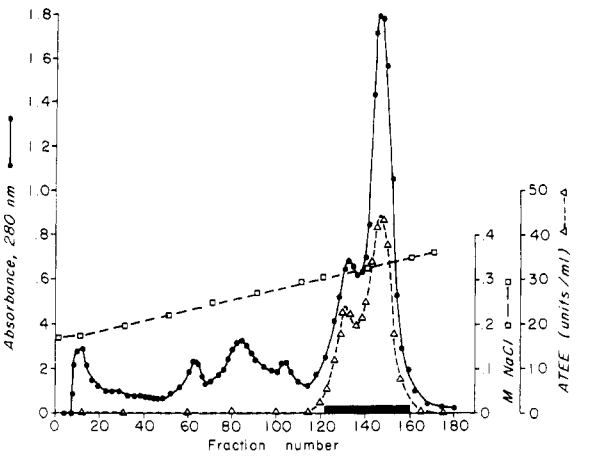

FIGURE 2: Chromatography on DEAE-Sephadex A-50 of the active enzyme fraction eluted from DEAE-cellulose (Figure 1). The column (2.5 × 30 cm) was developed at a flow rate of 30 ml/hr and 5-ml fractions were collected. Units of activity are μmoles of substrate hydrolyzed per min per ml of solution. Fractions indicated by a bar were pooled for subsequent gel filtration on Sephadex G-150.

Bromosuccinimide was from Arapahoe Chemicals, Inc. Iodoacetic acid, iodoacetamide, and rose bengal were purchased from J. T. Baker Chemical Co. Mercuribenzoate was obtained from Calbiochem. Phenylmethanesulfonyl fluoride and *N*-tosyl-L-phenylalanine chloromethyl ketone were from Cyclo Chemical Corp. Ethoxyformic anhydride and 1,2-epoxy-2-phenoxypropanewere purchased from Eastman Organic Chemicals. *N*-Acetylimidazole and phenylglyoxal were from K&K Laboratories, Inc. *N*-Ethylmaleimide was obtained from Schwarz Bioresearch Inc. Nitrophenylsulfenyl chloride was from Sigma Chemical Co. 2,4,6-Tribromo-4-methylcyclohexadienone was a gift from Dr. Yigal Burstein. *N*-Bromoacetyl-*N*-methyl-L-phenylalanine was a gift from Dr. G. M. Hass. Diazo-1*H*-tetrazole was synthesized according to Sokolovsky and Vallee (1966).

Methods

Amino Acid Composition. A Spinco Model 120 amino acid analyzer was used for all analyses. Unless otherwise noted, the amino acid composition was derived from triplicate analyses of protein samples which had been hydrolyzed in 6 N HCl *in vacuo* at 110° for 24, 48, and 96 hr.

Amino Terminal Sequence Analysis. A Beckman sequencer (Model 890) was used for the determination of the amino terminal sequences (Edman and Begg, 1967; Hermodson *et al.*, 1972a). The phenylthiohydantoin-amino acids were identified by gas chromatography with the exceptions of histidine and arginine which were identified by spot tests using diazotized 1-anisidine and phenanthrenequinone, respectively.

Samples were reduced and S-carboxymethylated before sequenator analysis as follows. The protein (5–10 mg) was dissolved in 5 ml of 1.0 M Tris-HCl (pH 8.0) containing 6 M guanidinium chloride. The solutin was adjusted to 0.1 M mercaptoethanol by the addition of 34 μl of the reagent (15 M) and allowed to stand at room temperature under nitrogen for 4 hr. The solution was then adjusted to 0.11 M iodoacetate by the addition of 1.2 ml of 0.5 M reagent. After 20 min, 34 μl of 15 M mercaptoethanol was added and the solution dialyzed in a rocking, flow-through dialysis apparatus against 20 l. of 5% acetic acid for 36 hr. All manipulations following the addition of iodoacetate were performed in the dark. After dialysis the protein sample was lyophilized, suspended in 33% acetic acid, and introduced into the reaction vessel of the sequencer.

Sodium Dodecyl Sulfate Gel Electrophoresis. The procedure of Weber and Osborn (1969) was used with the following modifications. Prior to electrophoresis, the protein samples were incubated for 2 hr at 37° in 0.01 M sodium phosphate (pH 7.0) containing 1% sodium dodecyl sulfate and 1% mercaptoethanol. This modification had no effect on the pattern obtained when compared to gels run as suggested by Weber and Osborn (1969). Instead of the original gel buffer, a buffer composed of 1.45 g of $NaH_2PO_4 \cdot H_2O$, 0.66 g of $Na_2HPO_4 \cdot H_2O$, and 2 g of sodium dodecyl sulfate diluted to 1 l. with distilled water was used (private communication of M. Pangburn).

After electrophoresis, the gels were severed at the front of the dye band and stained, and the mobilities were calculated by dividing the distance of migration by the length of the gel. This modification obviated the need for compensating for changes in gel size during staining and destaining.

Carbohydrate Analysis. Total hexose content was determined by the phenol–sulfuric acid method of Hirs (1967). The results were expressed in terms of 1:1 (w/w) galactose–mannose standard. Total amino sugar was determined by the Elson–Morgan method which was modified by Gatt and Berman (1966) using glucosamine hydrochloride as the standard. Neuraminic acid was determined by the Warren procedure (Warren, 1959).

Enzymatic Assays. Esterase activity was measured in a Radiometer TTT-1 autotitrator with 0.1 M NaOH as the titrant. Ac-Tyr-OEt (10 mM), dissolved in 0.01 M sodium phosphate–0.15 M NaCl (pH 8.0), was routinely used as the substrate.

Exopeptidase activity was determined with Cbz-Phe-Leu as the substrate. Hydrolysis was followed by measuring the decrease in absorbance at 224 nm of a 1 mM solution dissolved in 0.1 M sodium phosphate–0.15 M NaCl (pH 6.0).

Release of carboxyl terminal amino acids from N-substituted dipeptides was recorded continuously utilizing a Technicon autoanalyzer essentially as described by Lenard *et al.* (1965). Chart speed was set at 1 in./min. Portions of the reaction mixture were continuously withdrawn, at the rate of 0.33 ml/min, through Teflon tubing which passed through a boiling water bath and then into the analytical system of the analyzer. Substrates (0.25–10 mM) were dissolved in 0.05 M 2-(*N*-morpholino)ethanesulfonic acid–0.15 M NaCl (pH 6.0) containing 10% dioxane. The method was calibrated with standard solutions of the appropriate amino acids.

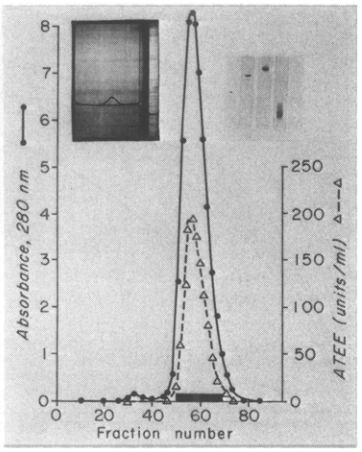

FIGURE 3: Gel filtration on Sephadex G-150 of the pooled fractions from DEAE-Sephadex chromatography (Figure 2). The column size was 2.5 × 120 cm. Fraction volume was 5 ml. The pooled fractions were precipitated by the addition of sufficient solid ammonium sulfate to reach 90% saturation. The precipitate was dissolved in 5 ml of elution buffer (0.07 M sodium phosphate (pH 7.0) containing 0.15 M NaCl). A flow rate of 25 ml/hr was maintained by the hydrostatic pressure of a 40-cm head. Right insert: analytical disc gel electrophoresis of protease C. Gels are from left to right: gel electrophoresis in the presence of mercaptoethanol and sodium dodecyl sulfate; pH 8.3 system containing urea; standard pH 8.3 system. Migration was from top to bottom (cathode to anode). Gels were severed at the dye front before staining. Left insert: Schlieren optical pattern of yeast protease C after 75-min centrifugation at 60,000 rpm. The protein concentration was 6.0 mg/ml, the bar angle 60°. Sedimentation was from right to left.

Heat Inactivation. Samples (0.5 ml) of a solution of protease C (0.1 mg/ml in 0.01 M sodium phosphate–0.15 M NaCl (pH 7.0)) were placed in test tubes which were sealed with parafilm. The tubes were placed in a constant-temperature bath at the desired temperature and, at appropriate times, removed and cooled in an ice bath, and peptidase activity was determined.

Effects of Urea. The enzyme was dissolved in 0.1 M sodium phosphate–0.15 M NaCl (pH 6.5) containing a predetermined concentration of urea. After incubation for 30 min at 25° peptidase activity was determined using substrate with or without urea.

pH Stability. Enzyme (1 mg/ml) was dissolved in buffers of 0.1 M sodium acetate (pH 3, 4, or 5), sodium phosphate (pH 6 or 7), or sodium borate (pH 8, 9 or 10) which had been adjusted to ionic strength 0.15 M. At zero time and after 2 hr aliquots were assayed for peptidase activity.

Results

Isolation and Purification of Yeast Protease C. Yeast protease C was purified by a modification of the procedure of Hata *et al.* (1967b). Compressed baker's yeast (24 lbs) was crumbled and mixed with 5 l. of chloroform. The mixture was occasionally stirred for 3 hr during which time the yeast became liquified. Excess chloroform was removed by aspiration, 6 l. of distilled water was added, and the pH was adjusted to 7.0 by the dropwise addition of 1.0 N NaOH. After the mixture was allowed to autolyze overnight at room temperature with constant stirring, cellular debris was removed by centrifugation. The supernatant (10.7 l.) contained no detectable esterase activity. The enzyme was activated by adjusting the supernatant to pH 5.0 by the dropwise addition of 1.0 N HCl and allowing the protein to undergo autolysis overnight at room temperature. The fully active autolysate was brought to pH 7.0 by the dropwise addition of 1.0 N NaOH and then to 90% saturation by the addition of solid ammonium sulfate while the pH was maintained at 7.0 with 1.0 N NaOH. The precipitate which formed overnight (at 4° with stirring) was collected by centrifugation at 20,000*g* for 30 min, dissolved in a minimal amount of water, and dialyzed against two changes of distilled water and finally against 0.01 M sodium phosphate buffer (pH 7.0) containing 0.15 M NaCl.

TABLE I: Purification of Yeast Protease C.

Step	Vol (ml)	Total Protein (mg)	Total Units	Specific Activity	Yield (%)
Autolysis at pH 7.0	10,650	238,560	0	0	
Autolysis at pH 5.0	10,650	213,596	37,275	0.175	100
90% ammonium sulfate	1,092	17,080	37,128	2.17	99.6
DEAE-cellulose	1,145	732.8	18,320	25.0	49.1
DEAE-Sephadex	184	353.2	16,560	47	44.4
Sephadex G-150	585	233.2	13,986	60	37.5

The dialyzed solution was applied to a DEAE-cellulose column (4.0 × 100 cm) which was previously equilibrated with 0.01 M sodium phosphate buffer (pH 7.0) and then developed by applying a linear gradient of NaCl (0.15–0.45 M) in the same buffer (Figure 1). Active fractions were pooled and the protein was precipitated by the addition of sufficient solid ammonium sulfate to reach 90% saturation. The precipitate was collected by centrifugation for 30 min at 20,000*g*, dissolved in a minimal amount of sodium phosphate buffer containing 0.15 M NaCl, and dialyzed overnight against the same buffer.

The dialyzed solution was applied to a column (2.5 × 50 cm) of DEAE-Sephadex A-50 which was previously equilibrated with 0.01 M sodium phosphate buffer (pH 7.0) containing 0.15 M NaCl. The column was developed by applying a linear gradient of NaCl (0.15–0.60 M) in the same buffer (Figure 2). Active fractions were pooled and the protein was precipitated with ammonium sulfate as before. The precipitate was dissolved in a minimal amount of phosphate buffer containing 0.15 M NaCl and dialyzed overnight against the same buffer. The solution was applied to a column (2.5 × 120 cm) of Sephadex G-150 which was previously equilibrated with 0.01 M sodium phosphate buffer (pH 7.0) containing 0.15 M NaCl. Fractions eluted from the column were tested for activity with the results shown in Figure 3. Fractions with constant specific activity were pooled, dialyzed against three changes of 6 l. of distilled water, and lyophilized. The solid protein was stored at −20°.

The purification and yields of the enzyme are summarized in Table I.

Physicochemical Characterization of Yeast Protease C. The homogeneity of the enzyme prepared in this manner was demonstrated by electrophoresis on polyacrylamide gels at in pH 8.3 in the presence of 8 M urea; a single narrow band was observed (Figure 3). The diffuse band in the absence of urea may be due to self-association of the enzyme. Supporting evidence for homogeneity was obtained by sedimentation in the ultracentrifuge. A sample of the lyophilized protein was dissolved and dialyzed against a potassium phosphate buffer (pH 6.5) of 0.1 ionic strength and examined in the ultracentrifuge (Figure 3). The symmetrical nature of the boundary (0.6% protein) suggests a high degree of homogeneity ($s_{20,w}$ = 4.6 S).

TABLE II: Amino Acid Composition of Protease C.

Amino Acid	Residues/Molecule[a]
Aspartic acid	68.5
Threonine[b]	19.5
Serine[b]	31.1
Glutamic acid	45.3
Proline	25.8
Glycine	36.2
Alanine	24.0
Valine[d]	31.6
Methionine[e]	6.6
Isoleucine[d]	19.7
Leucine	35.0
Tyrosine	23.2
Phenylalanine	23.5
Lysine	16.7
Histidine	8.0
Arginine	8.8
Tryptophan[f]	12.1
½ Cystine	13,[c] 12.6[e]

[a] Average values of nine analyses based on 24.0 mol of alanine/62,000. [b] Extrapolated to zero time of hydrolysis. [c] Determined as Cm-cysteine after reduction and alkylation. [d] Values after 96-hr hydrolysis. [e] Half-cystine and methionine were determined as cysteic acid and methionine sulfone, respectively, after performic acid oxidation. [f] Determined by the method of Edelhoch (1967).

Additional evidence for the homogeneity of protease C was obtained by polyacrylamide gel electrophoresis of the protein in the presence of sodium dodecyl sulfate and mercaptoethanol. The gel shown in Figure 3 revealed a single band corresponding in mobility to a molecular weight of 62,000.

Chemical purity was established by sequenator analysis which gave lysine as the sole amino-terminal residue. An extended analysis revealed the following amino terminal sequence: Lys-Ile-Lys-Asp-Pro-Lys-Ile-Leu-Gly-Ile-Asp-Pro.

The amino acid composition of yeast protease C is given in Table II. The value reported for half-cystine was obtained after reduction and carboxymethylation (13.6 residues/molecule). Performic acid oxidation yielded 12.6 residues of cysteic acid

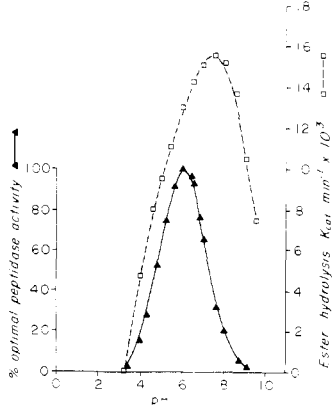

FIGURE 4: pH dependence of peptidase and esterase activities of yeast protease C. Peptidase activity is expressed as per cent of the maximum rate of hydrolysis of Cbz-Phe-Leu. The results of esterase hydrolysis (Ac-Tyr-OEt) are expressed as k_{cat}. Above pH 9.5, ester hydrolysis could not be determined due to base-catalyzed hydrolysis of the substrate.

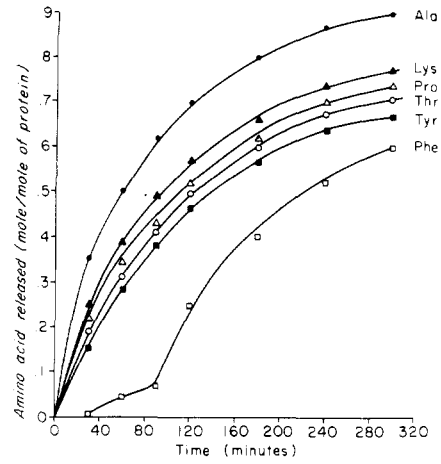

FIGURE 5: Time course of the release of amino acid residues from the B-chain of oxidized bovine insulin by yeast protease C. Protein concentration was 4 mg/ml in 0.1 N pyridine-acetate, pH 6.0. The reaction was performed at 25° with a substrate to enzyme ratio of 300. The results are in agreement with the actual sequence of -Phe-Tyr-Thr-Pro-Lys-Ala-OH.

per molecule. In the absence of denaturing agents, the protein failed to react with the Ellman reagent (Ellman, 1959) but in the presence of 8 M urea, 1.1 residue of thiol per protein molecule reacted (P. M. Levy, unpublished observations). These results agree with those reported by Hayashi *et al.* (1973).

The protein was found to be a glycoprotein containing 2.6% amino sugars and 12.7% neutral hexose, but no sialic acid.

Measurements of the stability showed that the enzyme was stable for 2 hr in the pH range 5.0–8.0 at 25° and between pH 5.0 and 7.0 at 37°. The enzyme was completely active when exposed for 5 min to 55° at pH 7.0; 50% of the activity was lost after 5 min at 62° while at 68° under these conditions, all activity was lost. A plot of ln k_1 (where k_1 is the first-order rate constant of inactivation) *vs.* $1/T$ was linear, the slope corresponding to a value of 73 kcal/mol for the heat of inactivation.

Concentrations of urea up to 4 M had no effect upon enzyme activity while at higher concentrations activity was rapidly lost. Preincubation in urea followed by assay in the absence of urea consistently yielded higher enzyme activity than when the enzyme was assayed in the presence of urea, suggesting that urea denaturation was reversible.

Enzymatic Properties. The pH dependence of esterase and peptidase activities is shown in Figure 4. Each activity reveals a

TABLE III: Hydrolysis by Yeast Protease C of N-Substituted L-Dipeptides of the Type Cbz-X-Leu.

Substrate	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m	Excess Substrate Inhibition
Cbz-Gly-Leu	364	2.95	123	–
Cbz-Phe-Leu	905	0.24	3,709	+
Cbz-Ala-Leu	27,723	1.28	21,658	–
Cbz-Val-Leu	848	0.10	8,230	+
Cbz-Leu-Leu	2,568	0.12	22,137	+
Cbz-Ile-Leu	901	0.10	8,743	+
Cbz-Ser-Leu	1,075	1.7	632	–
Cbz-His-Leu	84	1.8	47	–
Cbz-Pro-Leu	49	1.8	27	–
Cbz-Glu-Leu	1,792	8.3	216	–
Cbz-Nle-Leu	2,620	0.03	43,667	+

TABLE IV: Hydrolysis by Yeast Protease C of N-Substituted Dipeptides Of the Type Cbz-Gly-X.

Substrate	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m
Cbz-Gly-Leu	364	2.95	123
Cbz-Gly-Phe	135	1.2	116
Cbz-Gly-phe	Not hydrolyzed		
Cbz-Gly-Arg	40	16.3	2.4
Cbz-Gly-Glu	40	24.1	1.7
Cbz-Gly-Pro	Hydrolyzed but kinetics constants not measurable		
Cbz-Gly-Gly			
Acetyl-Gly-Leu	115	45.3	2.5

relatively sharp pH optimum with an apparent maximum at pH 6.0 for peptidase activity and at pH 7.5 for esterase activity.

The specificity of the enzyme was determined using the oxidized B chain of insulin as a substrate. Figure 5 shows that the enzyme released free amino acids from the carboxyl terminus in a sequential manner. In addition to aromatic and neutral amino acids, lysine and proline were released in nearly stoichiometric amounts. The ability of protease C to release acidic amino acids at pH 4.15 was previously demonstrated in the sequence analysis of monkey amyloid protein A (Hermodson *et al.*, 1972b).

The action of the enzyme on a series of N-substituted dipeptides reveals that at least three sites of the substrate influence the hydrolysis rates. An analysis of the hydrolysis of a series of *N*-carbobenzoxy dipeptides of the type Cbz-X-Leu, in terms of the kinetic parameters k_{cat} and K_m, is shown in Table III.

If the penultimate amino acid is an aromatic or a long chain hydrophobic residue, K_m is decreased by one order of magnitude as compared to substrates containing in this position alanyl, glycyl, or hydrophilic residues. Substrates containing more hydrophobic side chains can bind in a manner producing excess substrate inhibition. If one of the hydrogen atoms of glycine is replaced by a methyl group, a marked enhancement of K_{cat} is observed while K_m is essentially unchanged (compare Cbz-Gly-Leu with Cbz-Ala-Leu). Substitution of alanine by valine or leucine results in a decrease in both k_{cat} and K_m. If the penultimate amino acid is histidine or proline, k_{cat} is decreased by one order of magnitude as compared to Cbz-Gly-Leu while K_m is unchanged. The lowest K_m is observed for the peptide containing norleucine as the penultimate amino acid.

Variation of the carboxyl terminal amino acid also affects the kinetic constants (Table IV). If Cbz-Gly-Leu is used as the standard of comparison, substitution of either arginine (basic) or glutamic acid (acidic) for leucine reduces k_{cat} by one order of magnitude and increases K_m. The substitution of the aromatic residue phenylalanine for leucine affects neither parameter significantly.

Comparison of the rate of hydrolysis of Cbz-Gly-Leu with that of acetyl-Gly-Leu (Table IV) shows that varying the blocking group affects K_m more than k_{cat}.

Effect of Inhibitors. The ability of a number of different group-specific reagents to inhibit yeast protease C is documented in Table V. Typical sulfhydryl reagents, including $HgCl_2$, *p*-mercuribenzoate, and iodoacetamide, inhibit the enzyme but their reaction with residues other than cysteine (Webb, 1966) has not been excluded in this work. Reaction

TABLE V: Effect of Inhibitors on Yeast Protease C.[a]

Inhibitor	Probable Site of Action	Condition	Method
I. Inhibition			
p-Mercuribenzoate	Cys, His	1 mM[b]	Boyer (1954)
$HgCl_2$	Cys, His	1 mM[b]	Webb (1966)
1,2-Epoxy-3-phenoxypropane	Met, Glu, Asp	0.1 M[c]	Tang (1971)
Dip-F	Ser	1 mM[b]	Hartley (1960)
$PhCH_2SO_2F$	Ser	1 mM[b]	Gold (1967)
TPCK	His	2 mM[b]	Schoellman and Shaw (1963)
Phenylglyoxal	Arg	1.5%[d]	Takahashi (1968)
Photooxidation	Cys, Trp, His, Met, Tyr	Rose Bengal dye[b]	Abe *et al.* (1971)
Iodoacetamide	Cys, His, Lys	0.2 M[c]	Gurd (1967)
Ethoxyformic anhydride	His, Glu, Asp, Lys, Tyr	100-fold molar excess	Huc *et al.* (1971)
II. No Inhibition			
Iodoacetic	Cys, His, Lys	0.2 M[c]	Gurd (1967)
Nitrophenylsulfonyl chloride	Trp	100-fold molar excess[e]	Scoffone *et al.* (1968)
N-Bromosuccinimide	Trp, Met, Cys	100-fold molar excess[e]	Spande and Witkop (1967)
2,4,6-Tribromo-4-methyl-cyclohexadienone	Trp	100-fold molar excess[e]	Burstein and Patchornik (1972)
N-Bromoacetyl-*N*-methyl-L-phenylalanine	Cys, His, Lys, Tyr, $-COO^+$, Met, NH_2	1 mM[c]	Hass and Neurath (1971a)
Tetranitromethane	Tyr	50-fold molar excess	Sokolovsky *et al.* (1966)
N-Acetylimidazole	Tyr, His, Lys, Cys	50-fold molar excess	Simpson *et al.* (1963)
Diazo-1*H*-tetrazole	Tyr, His	50-fold molar excess	Sokolovsky and Vallee (1966)
N-Ethylmaleimide	Cys	1 mM[b]	Riordan and Vallee (1967)
N-(4-Dimethylamino-3,5-dinitrophenyl)maleimide	Cys	1 mM[b]	Witter and Tuppy (1960)

[a] Enzyme concentration, 1 mg/ml. [b] 0.1 M sodium phosphate–0.15 M NaCl (pH 7.0). [c] 0.1 M sodium phosphate–0.15 M NaCl (pH 6.5). [d] 0.1 M sodium phosphate–0.15 M NaCl (pH 7.5). [e] 0.25 M sodium acetate (pH 4.5). [f] 0.02 M sodium veronal–0.5 M NaCl (pH 7.5). [g] 0.25 M sodium acetate.

with typical tyrosine reagents fails to inactivate the enzyme even when up to five tyrosine residues per molecule are modified (tetranitromethane). Tryptophan-directed reagents (*N*-bromosuccinamide) also failed to cause inhibition after modification of three residues. The presence of carboxyl groups near the active site is suggested by the transient inhibition by ethoxyformic anhydride (Huc *et al.*, 1971), by the inhibition by 1,2-epoxy-3-phenoxypropane(Tang, 1971), and by the observation that the neutral alkylating agent iodoacetamide inhibits the enzyme while iodoacetate has no effect. Yet, *N*-bromoacetyl-*N*-methyl-L-phenylalanine, which reacts specifically with Glu_{270} of bovine carboxypeptidase A (Hass and Neurath, 1971b) and B (Hass *et al.*, 1972), has no effect on yeast protease C.

The presence of an active serine in the enzyme is indicated by the inhibition by Dip-F and phenylmethanesulfonyl fluoride. Total loss of activity is observed coincident with the incorporation of 0.98 mol of Dip-F/mol of enzyme, in agreement with the recent report by Hayashi *et al.* (1973). A serine residue is likewise the site of phosphorylation (R. W. Kuhn and H. Neurath, in preparation).

The enzyme is also inhibited by *N*-tosyl-L-phenylalanine chloromethyl ketone, by photooxidation in the presence of rose bengal and by phenylglyoxal (R. W. Kuhn and H. Neurath, in preparation). In contrast to the pancreatic carboxypeptidases, yeast protease C is not inhibited by the metal-chelating agents EDTA and 1,10-phenanthroline.

Discussion

The present procedure of isolating yeast protease C represents an improvement over that of Hayashi *et al.* (1973) for operation at a laboratory scale: reduction of solution volumes occurs at early stages, chromatography on DEAE-cellulose is more effective because of gradient rather than stepwise elution, and one DEAE-Sephadex column is eliminated. A double peak is routinely observed on DEAE-Sephadex (Figure 2), and attempts to distinguish between the two forms with regard to amino terminal sequence, amino acid composition, molecular weight, and kinetic parameters have been unsuccessful. Rechromatography on DEAE-Sephadex A-50 following dialysis and lyophilization yields only one peak of protein and enzyme activity. The enzyme isolated by this procedure is free of protease A, an endopeptidase which can easily contaminate the product, and appears to be homogeneous by several criteria. The enzyme contains an amino-terminal lysine residue in agreement with the observation of Aibara *et al.* (1971).

Yeast protease C releases a variety of free amino acids from proteins and synthetic *N*-carbobenzoxy dipeptides. Experiments with these dipeptides indicate that at least three sites of the substrate affect hydrolysis rates. Substrates containing charged amino acids in the carboxyl-terminal position yield higher K_m and lower k_{cat} values than those with hydrophobic residues. The type of residue in the penultimate position also is a major determinant of both K_m and k_{cat}. Substitution of alanine for glycine in the penultimate position results in a marked increase in k_{cat}. The effect upon K_m produced by varying the nature of the blocking group suggests that this site of the substrate also influences hydrolysis rates. Experiments with the oxidized B chain of bovine insulin indicate that the enzyme sequentially releases free amino acids from the carboxyl-terminus. Particularly noteworthy is the release of proline. These experiments also revealed that the enzyme preparation was free of any contaminating endopeptidase activity.

Although no homology could be detected between the amino terminal sequence of yeast protease C and any other protease, the enzyme does share common catalytic features with both the metallocarboxypeptidases and the serine proteases. Whereas the enzyme is not inhibited by chelating agents, and thus is not a metalloenzyme, the inhibition by phenylglyoxal implicates the presence of an arginine at or near the active site. This residue may be involved in binding the carboxyl group of peptide substrates as in the case of bovine carboxypeptidase A (Lipscomb *et al.*, 1969). The catalytic mechanism has features in common with the serine proteases. The inhibition by Dip-F and phenylmethanesulfonyl fluoride indicates the presence of an active serine, while inhibition by *N*-tosyl-L-phenylalanine chloromethyl ketone implicates a histidine residue at the active site.

Aibara *et al.* (1971) have found that a free sulfhydryl group is required for enzyme activity. This interpretation is based largely on the inhibition by *p*-mercuribenzoate and by $HgCl_2$ and on reaction with 5,5′-dithiobis(2-nitrobenzoic acid) and is consistent with the present observations of inhibition by several sulfhydryl reagents.

The stability of the enzyme in urea solutions and its ability to release a wide variety of carboxyl-terminal amino acids from proteins and peptides at both neutral and acidic pH values render it highly useful for sequence determination (Hermodson *et al.*, 1972b).

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Reaction of Pyridoxal 5′-Sulfate with Apoenzyme of Aspartate Aminotransferase. Covalent Labeling of the Protein with Elimination of Sulfate†

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ABSTRACT: The 5′-sulfate esters of pyridoxine, pyridoxal, and pyridoxamine have been prepared and their absorption spectra and dissociation constants have been determined. Spectra have been resolved using log normal distribution functions. The pK_a values are compared with those of related compounds. That for the pyridinium group is strikingly lower in pyridoxal sulfate than in pyridoxal phosphate. When pyridoxal sulfate reacts at pH 8.3 with apoaspartate aminotransferase it slowly forms an absorption band at 24.9 kK (402 nm) which is narrow and displays marked vibronic fine structure. (This is in sharp contrast to the broader band at 27.4 kK [364 nm] formed with pyridoxal phosphate.) At lower pH the peak shifted to 25.9 kK (386 nm) and at higher pH to 27.4 kK. The pyridoxal sulfate–enzyme form absorbing at 24.9 kK has positive circular dichroism (like the native enzyme). Upon denaturation with acid the chromophore precipitated with the protein suggesting covalent attachment. Cysteine, β-mercaptoethylamine, ethylenediamine, ethanolamine, serine, and arginine all react with pyridoxal sulfate with gradual production of narrow absorption bands similar to that formed with the apoenzyme. Investigation of the reaction with cysteine reveals that inorganic sulfate is eliminated during the reaction at a rate that is identical with that of formation of the 24.9-kK chromophore. Investigation with nmr shows that formation of a thiazolidine ring is followed by loss of the 4′ proton and disappearance of the 5′-CH_2 peak. Elimination of sulfate to a quinonoid form that tautomerizes to a cyclic substituted Schiff base is proposed. It is speculated that an unknown group X in the enzyme replaces the SH group of cysteine in a similar reaction.

One approach to understanding the function of the phosphate group in the coenzyme pyridoxal 5′-phosphate (pyridoxal-P)[1] is to study analogs of similar geometry and charge type but containing different anionic side chains. Thus, replacement of the phosphorylated hydroxymethyl side chain of pyridoxal-P with a propionic acid side chain (5-deoxy-5′-carboxymethylpyridoxal) yields an analog of the coenzyme that binds to several apoenzymes. However, the activity with apo aspartate aminotransferase (apoAAT) is at most 3% of that of

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[1] Abbreviations used are: AAT, aspartate aminotransferase; pyridoxal-P, pyridoxal 5′-phosphate.