Carboxypeptidase from Yeast

LARGE SCALE PREPARATION AND THE APPLICATION TO COOH-TERMINAL ANALYSIS OF PEPTIDES AND PROTEINS*

(Received for publication, November 7, 1972)

RIKIMARU HAYASHI,† STANFORD N. MOORE, AND WILLIAM H. STEIN
From The Rockefeller University, New York, New York 10021

SUMMARY
In order to render the carboxypeptidase from yeast more readily available for use in sequence studies in protein chemistry, the procedure of Hata, Hayashi, and associates for the preparation of the enzyme has been adapted to a larger scale through cooperation with the New England Enzyme Center. From 100 pounds of bakers' yeast, the yield was about 1.4 g of the chromatographically purified, electrophoretically homogeneous enzyme, free of other proteinase activity. The carboxypeptidase from the yeast of different manufacture has properties corresponding very closely to those observed initially by Hayashi and Hata; the glycoprotein has a molecular weight of about 61,000, has a nitrogen content of 12.74%, yields 15.1 g of hexose per 100 g, and consists of a single polypeptide chain of 442 residues with 16 residues of glucosamine in the carbohydrate moiety. Lysine is at the NH₂ terminus and -Asp-Ser-Thr-Leu is the COOH-terminal sequence.

The broad carboxypeptidase activity at pH 5, which includes the liberation of proline, has been tested with the present preparation on glucagon, the B chain of insulin, and reduced and carboxymethylated pancreatic ribonuclease. The amino acids in these sequences released most slowly were glycine and aspartic acid. The carboxypeptidase retains activity in 6 M urea and can be used for the study of proteins that may have inaccessible COOH termini in the usual aqueous solution; native pancreatic ribonuclease is one example.

Several carboxypeptidases from nonmammalian sources are known to have the ability to remove most amino acid residues, including that of proline, from the COOH termini of proteins. The list includes enzymes from yeast (1-6), citrus peel (7), or citrus leaves (8, 9), bean leaves (10, 11), Aspergillus (12), and cotyledons of germinating cotton seedlings (13, 14). The carboxypeptidases from most of these sources have the property of being inhibited by diisopropyl phosphofluoridate, as is also true of a carboxypeptidase from germinating barley (15), an enzyme which is not reported to release proline. In each instance, removal of possible contaminating traces of proteinases is a key aspect of the method of preparation. The enzyme from yeast has properties which facilitate the scaling-up of the preparation and the isolation of a homogeneous enzyme.

The present experiments are based upon the procedures of Hata, Hayashi, and associates (2-6, 16, 17) for the isolation from bakers' yeast of a purified glycoprotein (originally termed proteinase C) with carboxypeptidase activity. The enzyme, after formation from its precursor (16), is similar in its chromatographic properties to peptidase β isolated from brewers' yeast by Félix and Brouillet (1). These peptidases also act on terminal ester groups (as in N-acetyl-L-tyrosine ethyl ester (2)) or on terminal amide groups (as in benzoylcarbonyl-glycyl-L-phenylalanine amide (1, 4)), but the rate of hydrolysis is much slower for the amides than for terminal groups with the —COOH free. Since the principal action of these enzymes is in the removal of COOH-terminal residues from polypeptide chains, it is practical to term them carboxypeptidases; in an analogous sense, chymotrypsin is generally classified as a proteinase rather than as an esterase. The designation carboxypeptidase Y can serve to distinguish the yeast enzyme from the similar enzymes from other sources.

EXPERIMENTAL PROCEDURE

Materials—Fleischmann's compressed bakers' yeast was purchased as 1-pound cakes from Standard Brand's Inc., New York. Bovine pancreatic RNase A (RAF grade) and carboxypeptidase A (COADFP grade) were obtained from Worthington; crystalline porcine glucagon was obtained from Lilly and the B chain of RCM bovine insulin was purchased from Schwarz-Mann. The RCM RNase used was a sample prepared by Crestfield et al. (18). Benzoylcarbonyl dipetidyl peptides were purchased from Cyclo; α-N-acetyl-L-tyrosine ethyl ester and α-N-benzoyl-L-tyrosine p nitroanilide from Sigma. DFP was purchased from Aldrich.

Assay of Enzymatic Activity—The activity of carboxypeptidase Y was assayed in three ways. In the course of purification of the enzyme, activity against α-N-acetyl-L-tyrosine ethyl ester (2) was measured with a Radiometer pH-stat. Localization of the enzyme in chromatographic eluates was conveniently performed

* This study was supported in part by Grant CM 07256 from the United States Public Health Service.
† Present address, The Research Institute for Food Science, Kyoto University, Kyoto, Japan.
by measurement at 410 nm of the product of the hydrolysis of α-N-benzoyl-L-tyrosine p-nitroanilide (17). Carboxypeptidase activity of the purified enzyme was determined by measuring the rate of release of free amino acid from benzoylcarbonyl-L-glutamyl-L-L-tyrosine or benzoylcarbonyl-L-phenylalaninyl-L-leucine. The reaction mixture was 3.3 mM in substrate and 50 mM in sodium acetate buffer or pyridine acetate buffer, at pH 5.5 in a total volume of 1 ml. The reaction mixture was incubated at 25° for 10 or 20 min. After incubation, the reaction was terminated by the addition of ninhydrin reagent (19), and the color developed was estimated after activation of the precursor (16) in 25% dioxane. The proteinase activity of yeast and subsequent fractions was determined against casein (Penetz) by a modification (2) of the method of Kunitz.

Measurements of Carboxypeptidase Activity—With glucagon and the B chain of RCM insulin, 0.8 mg of substrate was dissolved in 200 µl of 0.1 M pyridine acetate buffer (pH 5.5), and a few µl of solution containing 8 µg of carboxypeptidase Y were added. Incubation was at 35° for 2 hours. The hydrolysis was stopped by heating the mixture in a boiling water bath for a few minutes. The pyridine acetate buffer was removed in a desiccator; the supernatant, after correction for moisture content (a separate sample was dried overnight at 105° to constant weight), was then used for amino acid analysis after the removal of any insoluble material by centrifugation. With RCM RNase as substrate, the sample was 1.35 mg and 1% of that amount of enzyme was used for digestion at 25° for 15 hours.

Unmodified RNase (10 mg) was digested at 25° by 45 µg of carboxypeptidase Y in 1 ml of the pH 5.5 buffer which was made 6 M in urea and contained 200 mmoles of noreneucleic acid as internal standard. The reaction was terminated by adding Dowex 50-X2 (H+ form, 120 mesh) until the pH of the supernatant, after shaking, was 2.5 to 3. The adsorbed amino acids were eluted by 5 M H4O1 according to the procedure of Ambler (21) and measured on an automatic analyzer.

COOH-terminal and NHz-terminal Analysis of Carboxypeptidase Y—COOH-terminal analysis of carboxypeptidase Y was performed with carboxypeptidase A because denatured carboxypeptidase Y was insoluble at pH 5.5 but soluble at about pH 8. Freshly prepared carboxypeptidase Y was inactivated by incubation at room temperature for 30 min with 100 mM sodium phosphate (pH 7.0), 1 mM in DFP. The reaction mixture was desalted on a column of Sephadex G-25 with 30% acetic acid as the eluent, and the protein fraction was lyophilized. The protein (1.2 mg) was dissolved in 1 ml of 100 mM N-ethylmorpholine acetate (pH 7.6) and heated in a boiling water bath for 5 min. Carboxypeptidase A (20 µg) was added to the cooled solution and the mixture was incubated at 25°. At appropriate time intervals, 200-µl portions of the mixture were withdrawn and treated as described for the carboxypeptidase Y digestion of RCM RNase.

NH2-terminal residues were estimated by the cyanate method (28), modified for application to about 1 mg of carbamylated protein. Chromatographic analysis was performed on the nanomole scale. Values were corrected by the recovery factors given by Stark and Smyth (29). Analysis of fraction C was omitted. Edman degradation by the subtractive method was performed as described by Salnikow et al. (30).

Amino Acid Analyses—Analyses of acid hydrolysates (22) were performed with a Durrum D-500 amino acid analyzer (range 1 to 10 nmoles per amino acid), and those of enzymic digests were made with a Beckman model 120 analyzer (range 5 to 30 nmoles) or in the 1-nmole range on an instrument of the Spackman et al. (23), type modified for use with 0.28-mm diameter columns by Dr. Ta-hsiu Liao of this laboratory. Tryptophan content was determined by the method of Hirs (36). Hexosamine content was estimated as glucosamine on an amino acid analyzer after the glycoprotein was hydrolyzed with 5.7 N HCl at 100° for 6 hours according to Johansen et al. (27).

Additional Methods—Disc electrophoresis (31) was performed with 7% polyacrylamide gel at pH 5.9 without a stacking gel. Molecular weight was estimated by disc electrophoresis in the presence of sodium dodecyl sulfate by the method of Weber and Osborn (32).

Total hexose was determined by the ornithin method of Tsugita and Akabori (33). Protein and nucleic acid content of the fractions obtained during the purification of carboxypeptidase Y were estimated by the spectrophotometric method of Warburg and Christian (34). Concentration of purified carboxypeptidase Y was determined spectrophotometrically by use of E280 nm — 15.0; this value was obtained with a sample of the purified enzyme after correction for moisture content (a separate sample was dried at 105° to constant weight). In the use of the enzyme on proteins, the substrate concentration was usually determined by amino acid analysis after acid hydrolysis of an aliquot of the protein solution.

Preparation of the Enzyme—An outline of the purification steps, which represent a scale-up and modification of those developed by Hayashi et al. (16) and Aibara et al. (17) is shown in Table I; Steps 1 to 5 were designed and conducted in cooperation with Mr. Henry Blair and Dr. Stanley E. Charn of the New England Enzyme Center, Tufts University School of Medicine, Boston. In Step 1, 100 ml of chloroform per pound of yeast (half of the volume used previously (16)) were found to be enough to cause solubilization of all of the potential enzyme activity. This change facilitated the centrifugation by reducing the amount of chloroform in suspension and decreased the extraction of nucleic acid.

The removal of cell debris and the fractionation by ammonium sulfate were critical steps in the large scale preparation because of the volumes that needed to be centrifuged at high speed. These steps were successfully accomplished through use of a Sharples centrifuge, the center cylinder of which was previously cooled to 5°. Antifoam (Dow Corning AF) was added for prevention of surface inactivation of the enzyme through foaming of the solution. The bottom part of the settled autolysate, which contained more chloroform than the top part, was centrifuged, separately, batchwise, in a bucket-type centrifuge.

For activation of the inactive precursor by proteolysis, (NH4)2SO4 precipitate obtained in Step 3 was dissolved in 3 liters of 30 mM sodium acetate buffer (pH 5.0) per kg of wet precipitate. Incubation at 25° and pH 5.0 for 20 hours yielded maximum activity (36). There is extensive digestion of undesired proteins in Step 4 by yeast proteinases as well as by the carboxypeptidase during the activation step, and the peptides are removed by the dialysis in Step 5.

For removal of nucleic acids and basic proteins, stepwise chromatography on diethylaminoethyl cellulose (DE 52, Whatman) was employed. The fractions containing enzyme coincided with the elution of a darkly colored eluate; the first eluate, light yellow in color, was discarded, and the next eluate, dark
**Table I**

**Purification of carboxypeptidase Y from yeast**

| Step | Volume or weight | Protein | Specific activity | Yield |
|------|-----------------|---------|------------------|-------|
| Step 1. Autolysis (16, 35) | 96 lbs | | | |
| Fresh Fleischmann's compressed bakers' yeast. Knead with 9.6 liters of chloroform at 25° (hood) and stir for 30 min after yeast has liquified. Add 19.2 liters of H₂O and stir thoroughly. Adjust to pH 7.0 with 1 N NaOH (ca. 2 liters). After the suspension has been at 25° for ca. 2 hrs, readjust to pH 7.0 with 1 N NaOH (ca. 380 ml). Let stand at 25° for 18 hrs. | | | |
| Step 2. Removal of cell debris | 70 liters | | | |
| Autolyzed mixture. Add antifoam (Dow Corning AF, 10 ml). Centrifuge top 9 parts in a Sharples model 16 (13,000 X g, 60 liters per hr) and the bottom part in an International PR-2 (2400 X g, 20 min). Centrifuge the combined supernatant solutions in the Sharples (13,000 X g, 20 liters per hr). | | | |
| Step 3. Fractionation by ammonium sulfate | 36 liters | 476 | 0.22 | 100 |
| Supernatant. Add 10.6 kg of ammonium sulfate at pH 7.0 (50% saturation). Add antifoam (10 ml). Centrifuge in Sharples (13,000 X g, 20 liters per hr). Discard precipitate. Add 11.9 kg of ammonium sulfate to supernatant at pH 7.0 (90% saturation). Centrifuge in Sharples (13,000 X g, 20 liters per hr). | | | |
| Step 4. Activation of the precursor | 2.2 kg (wet weight) | | | |
| Precipitate. Dissolve in 6.6 liters of 0.05 M sodium acetate (pH 5.0) and adjust to pH 5.0 with 1 N acetic acid (ca. 250 ml). Incubate at 25° for ca. 1 hr. Remove precipitate in Sharples, batchwise (13,000 X g, 20 min). Readjust to pH 5.0 and add 2 ml of toluene. Incubate at 25° for 10 hrs. | | | |
| Step 5. Stepwise chromatography | 8.5 liters | 180 | 1.06 | 91 |
| Activated enzyme solution. Adjust to pH 7.0. Dialyze in about 1-m sections of Visking tubing (size 36) in a 200-liter tank against three batches of 0.01 M sodium phosphate (pH 7.0), stirred by N₂, at 4° over a period of 2 days. Add NaCl to make the solution 0.1 M in NaCl. Apply the solution to a column of DE-52 (10 X 50 cm) equilibrated with 0.01 M sodium phosphate (pH 7.0), 0.1 M in NaCl (flow rate, 1 liter per hr). Wash the column with 10 liters of the starting buffer. Change to the same buffer 0.3 M in NaCl. First 2 liters (light yellow) contain no activity. Collect the active fractions (dark yellow). Precipitate the enzyme by dialysis against two batches of saturated (NH₄)₂SO₄, adjusted to pH 7.25, 25 liters, at 4° over a period of 2 days. Collect precipitate by centrifugation with International HR-1 (38,000 X g, 60 min). | | | |
| Step 6. Chromatography on DEAE-Sephadex A-50 | 580 ml | 1.6 | 88 | 64 |
| Precipitated enzyme. Dissolve in minimum volume of 0.01 M sodium phosphate (pH 7.0), 0.1 M in NaCl and dialyze against two batches of this buffer (overnight). Apply to column of DEAE-Sephadex (2.6 X 43 cm) equilibrated with the same buffer. Elute with a linear increase in NaCl concentration from 0.1 to 0.42 M (each chamber 1000 ml). Flow rate, 100 ml per hour (See Fig. 1). | | | |
| Step 7. Rechromatography | 460 ml | 1.4 | 90 | 57 |
| Pooled active fractions (Fig. 1). Precipitate the enzyme by dialysis against saturated (NH₄)₂SO₄, as in Step 5 (volume, 6 liters). Centrifuge in a Sorvall RC2-B (27,000 X g). Dissolve precipitate and dialyze as in Step 6. Apply to column of DEAE-Sephadex (2 X 37 cm). Elute with a linear increase in NaCl concentration from 0.2 to 0.42 M (each chamber 600 ml). Flow rate, 20 ml per hour. | | | |

*a Adapted from References 16 and 17.

*b Micromoles of N-acetyl-L-tyrosine ethyl ester hydrolyzed per min per mg of protein.

*c Activity was determined after activation by 33% dioxane (6).
brown in color was collected. In this step, nucleic acids were almost completely removed and the yield of the enzyme was 80%.

On the large scale, precipitation of the enzyme by dialysis against saturated (NH₄)₂SO₄ was used. On a small scale preparation, ultrafiltration at 4° was used to concentrate the enzyme without detectable inactivation.

Final purification was performed by column chromatography on DEAE-Sephadex A-50 at 4° as described previously (2). After rechromatography, the enzyme was reprecipitated by dialysis against saturated (NH₄)₂SO₄. The thick suspension can be stored at −20° indefinitely. For use, a portion corresponding to about 100 mg of protein was dissolved in a minimum volume of water and dialyzed against H₂O or 0.01 M phosphate buffer (pH 7) to give 10 ml of an approximately 1% aqueous solution. About 1 ml portions of this solution were stored in vials at −20°. The frozen solutions showed no loss of activity in six months. Lyophilization causes loss of more than 20% of the activity.

**RESULTS**

**Purification and Characterization of the Enzyme**—The elution pattern for the first chromatography at 5° on DEAE-Sephadex A-50 is shown in Fig. 1. The content of proteinase A in the extract of Fleischmann's yeast was about half that obtained earlier with Oriental yeast (see Fig. 3 in Reference (2)), whereas the content of carboxypeptidase Y was about the same with the two commercial yeasts. Upon rechromatography, the carboxypeptidase yielded a single peak, and the specific activity was constant across the peak. From 96 pounds of yeast, 1.4 g of carboxypeptidase Y are obtained (yield, 57%). In laboratory scale experiments starting with 5 pounds of yeast, 45 mg of the purified enzyme were obtained (recovery, 30%). The degree of purification (about 450-fold) and the yield are calculated from activities measured after an aliquot from Step 2 is treated to convert fully the precursor to enzyme; otherwise, the calculation of the degree of purification comes out incorrectly high.

**Hydrolysis of Small Peptide Substrates**—The specific activities of the enzyme against benzoylcarbonyl-L-glutamyl-L-tyrosine and benzoylcarbonyl-L-phenylalanyl-L-leucine were 15.4 and 122 pmol min⁻¹ mg⁻¹, respectively. The latter peptide was the best substrate so far tested for the enzyme (Km ≤ 10⁻⁴ M). This enzyme hydrolyzed α-N-acetyl-L-tyrosine ethyl ester with a specific activity of 90 pmol min⁻¹ mg⁻¹. All of these activities were completely inhibited by 10⁻⁴ M DFP or p-HMB.

**Action on Larger Peptides and Proteins**—The enzyme hydrolyzed bovine glucagon, the B chain of RCM insulin, and RCM RNase releasing only the amino acids which are to be expected details and the assay procedures are described in Table I and under "Experimental Procedure."

**Fig. 1 (left).** First chromatography on a column (2.6 X 45 cm) of DEAE-Sephadex A-50 (cf. Reference 2). The initial buffer was 0.01 M sodium phosphate (pH 7.0), 0.1 M NaCl. Proteinase activity was measured against casein; carboxypeptidase Y was assayed photometrically by its amidase activity against α-N-benzoyl-L-tyrosine-p-nitroanilide. The final and largest peak represents carboxypeptidase Y. The fractions marked by a horizontal arrow were pooled for rechromatography. Other experimental results are in full agreement with earlier ultracentrifugal measurements (17).
from the COOH-terminal sequences, as shown in Table III. Neither peptides nor other amino acids were detectable on the amino acid analyzer. The results with insulin and RNase show that the glycine residues in these sequences are only slowly released. Aspartic acid was rapidly released from RCM RNase, whereas its release was slow from glucagon. With RCM RNase, hydrolysis of peptide bonds on both sides of a proline residue was achieved.

The above results, as well as the following observation, show that proteinase action, which might hydrolyze internal bonds in a protein to yield large peptide fragments, is absent in the preparation of carboxypeptidase Y. After an extensive digestion of RCM RNase by the enzyme (under the same conditions as used for the data in Table III), one aliquot of the digestion mixture was subjected to determination of the released amino acids and the other to one cycle of subtractive Edman degradation. It was found that within experimental error no amino acids other than NH₂-terminal lysine were lost by Edman degradation. In addition, no peptides of an intermediate size were found when the digestion mixture was subjected to gel filtration chromatography on Sephadex G-75 using 50% acetic acid as eluant.

Use for Analysis of COOH-terminal Sequences—In order to test the use of carboxypeptidase Y for sequence determination, the rates of release of amino acids from RCM RNase by the enzyme were followed quantitatively. The result is summarized in Fig. 4. In the first 10 min, five amino acids were liberated rapidly; thereafter histidine and proline appeared, accompanied by further release of valine. The results are consistent with the known structure of RNase (39, 40). Carboxypeptidase Y could not release any measurable amino acids from native RNase after a period of 30 min of incubation. However, the carboxypeptidase is still active in the presence of 6 M urea (6) and in such a solution released seven amino acids from RNase (Fig. 5). The rate of the release of amino acids was about half of that observed in the RCM RNase experiments. This method provides an approach to the determination of the COOH-terminal sequence of proteins that are difficult to denature. In these experiments, the control, containing only carboxypeptidase Y, yielded no detectable amino acids.

**DISCUSSION**

Yeast proteinase A is an enzyme which may contain carboxypeptidase Y, because both enzymes have similar properties (sugar content, isoelectric point, and molecular weight) (2). A specific inhibitor for proteinase A has not yet been found. However, the content of proteinase A in the extract of *Fleischmann's* yeast used here was remarkably smaller than that found with the Oriental yeast used previously (2). This fact facilitated the isolation of carboxypeptidase Y in pure form as judged by the absence of measurable proteinase activity when tested against glucagon, B chain of insulin, and RCM RNase. Hugli (41) has shown that glycine residues in these sequences are only slowly released. Aspartic acid was rapidly released from RCM RNase, whereas its release was slow from glucagon. With RCM RNase, hydrolysis of peptide bonds on both sides of a proline residue was achieved.

In the RCM RNase experiments, this method provides an approach to the determination of the COOH-terminal sequence of proteins that are difficult to denature. In these experiments, the control, containing only carboxypeptidase Y, yielded no detectable amino acids.

**FIG. 3. Rate of release of amino acids from DFP-inactivated carboxypeptidase Y by digestion with pancreatic carboxypeptidase A.** Protein concentration was 0.1% in 0.1 M N-ethylmorpholine-acetate (pH 7.6), and the reaction was carried out at 35°C with a substrate to enzyme ratio of 50. Other details are given in the text.

**TABLE II**

Amino acid composition of carboxypeptidase Y

| Residues | Contenta | Residues per moleculeb | Nearest integer |
|----------|----------|------------------------|----------------|
| Aspartic acid | 1.075 | 61.7 | 65 |
| Threoninec | 0.300 | 18.0 | 18 |
| Serinec | 0.560 | 33.6 | 31 |
| Glutamic acid | 0.686 | 41.1 | 41 |
| Proline | 0.415 | 24.9 | 25 |
| Glycine | 0.560 | 33.6 | 31 |
| Alanine | 0.414 | 24.8 | 25 |
| Half-cystinec | 0.188 | 11.3 | 11 |
| Valine | 0.495 | 29.7 | 30 |
| Methioninec | 0.121 | 7.2 | 7 |
| Isoleucine | 0.331 | 19.8 | 20 |
| Leucine | 0.613 | 36.8 | 37 |
| Tyrosine | 0.396 | 23.7 | 24 |
| Phenylalanine | 0.445 | 26.7 | 27 |
| Lysine | 0.310 | 18.0 | 19 |
| Histidine | 0.154 | 9.2 | 9 |
| Arginine | 0.145 | 8.7 | 9 |
| Tryptophanc | 0.176 | 10.6 | 11 |
| Total | 442 |

a The results are similar to those obtained with earlier preparations from a different brand of yeast (17). The values are the average of analyses of two hydrolysates obtained after 22 hours of hydrolysis in 6 M HCl in vacuo at 110°C and are based upon the dry weight of the glycoprotein (nitrogen 12.74%).

b Calculations were based on a molecular weight of 61,000 (17).

c Values for threonine and serine were corrected for 5 and 10% destruction, respectively, during hydrolysis (22).

d Half-cystine and methionine were determined as cysteic acid and methionine sulfone, respectively, after performic acid oxidation (28).

The NH₂ content of the hydrolysate, corrected for 5 and 10% destruction of threonine and serine, respectively, was 1.02 umoles per mg, corresponding to 62 amide-NH₂ groups per molecule. The total analysis, including glucosamine, accounts for 103.3% of the nitrogen of the glycoprotein.
TABLE III
Digestion of glucagon, RCM insulin B chain, and RCM RNase by carboxypeptidase Y

The substrates, at a concentration of about 0.5%, were digested at pH 5.5 at an enzyme to substrate ratio of 1:100. Glucagon and the B chain of insulin were incubated at 35° for 2 hours; RCM RNase was held at 25° for 15 hours. The released amino acids are expressed as mole per mole of substrate.

| Substrate                        | Released Amino Acids (mole per mole of substrate) |
|----------------------------------|--------------------------------------------------|
| Glucagon: Arg-Ala-Gln            | 0.07 0.69 0.85 0.84 0.92 0.81 1.4 0.75          |
| Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr | (37) |
| RCM insulin B chain: Cys(Cm)-Gly-Glu-Arg-Gly- | 0.04 0.14 0.04 0.10 |
| Phe-Phe-Tyr-Thr-Pro-Lys-Ala      | 1.42 0.88 1.0 1.0 0.80 1.0  |
| RCM RNase: Cys(Cm)-Glu-Gly       | 0.08 0.03 |
| Asn-Pro-Tyr-Pro-Val-His-Phe-Asp-Ala-Ser-Val | (39, 40) |
| Glu                  | 0.53                |
| Asp + Gln.                  |
| Phe + Tyr.                  |
| His + Pro.                  |

* Asn + Gln.
* The rate of hydrolysis slowed at Gly in these two chains.
* Ser + Asn.

FIG. 5. Rate of the release of amino acids from native RNase by digestion with carboxypeptidase Y in the presence of 6 M urea. Protein concentration was 1% in 0.1 M pyridine-acetate (pH 5.5), and the reaction was carried out at 25° with a substrate to enzyme ratio of 222. The results are consistent with the sequence -(Val, Pro)-His-Phe-Asp-Ala-Ser-Val (39, 40).

FIG. 4. Rate of release of amino acids from RCM RNase by digestion with carboxypeptidase Y. Protein concentration was 1% in 0.1 M pyridine-acetate (pH 5.5), and the reaction was performed at 25° with a substrate to enzyme ratio of 280. The results indicate the sequence -(Val, Pro)-His-Phe-Asp-Ala-Ser-Val (39, 40).

the presence of proteolytic enzymes since DNase is so susceptible to proteolysis (42).

Repeated freezing and thawing of solutions of carboxypeptidase Y, or prolonged storage of the enzyme at room temperature, can lead to autodigestion with the liberation of free amino acids, primarily the amino acids in the COOH-terminal portion of the protein. Extremely dilute solutions of the enzyme lose activity. Carboxypeptidase Y is relatively stable in the presence of protein denaturants (6); about 20% of the activity was lost after incubation with 6 M urea at 25° for 1 hour.

The specificity of carboxypeptidase Y has been examined with synthetic peptides (3). The enzyme released most types of amino acids, including proline, from the COOH termini of the substrates tested; however, the release of glycine and sometimes aspartic acid was slow. The latter observation is in accord with the result that poly-L-aspartic acid is a poor substrate for the enzyme, whereas poly-L-glutamic acid is rapidly hydrolyzed by the enzyme (4). The slow release of glycine is a property shared by pancreatic carboxypeptidase A (21), but the chemical properties of the yeast enzyme are markedly different from those of the pancreatic enzyme since carboxypeptidase Y is inhibited by DFP, p-HMB, and Hg²⁺.

The broad specificity and the stability in urea of carboxypeptidase Y make the enzyme applicable to some sequence determinations which are not possible with other carboxypeptidases. Furthermore, as a result of its amidase action, this enzyme might be applied to the sequence analysis of peptides having amidated COOH-terminal groups such as oxytocin and vasopressin. The preparation described in this communication has been used by Liao et al. (44) in sequence studies on peptides from pancreatic deoxyribonuclease (peptides number C4, Th20, Th8a, and T12-Th3b). Homoserine and carboxymethyl cysteine were released by carboxypeptidase Y, and the release of proline was helpful in two of the instances. Hermodson et al. (45) have used their own preparation of yeast carboxypeptidase to deduce the COOH-terminal sequence of amyloid protein A; aspartic acid was readily released in their experiments.

Acknowledgments—The generous cooperation of Mr. Henry Blair and Dr. Stanley E. Charm of the New England Enzyme Center was essential for the large scale experiments. The authors are indebted to Dr. Tony E. Hugli for advance information.

* In a recent communication, Shaw and Wells (43) have studied the reaction of DFP with a partially purified peptidase from bakers' yeast prepared by a modification of the procedure of Felix and Brouillet (1) for peptidase 8. The active DFP-sensitive enzyme component, which probably is the same as carboxypeptidase Y, was concluded to have the active serine residue in the sequence Glu-Ser-Tyr.
tion on the results of his use of carboxypeptidase Y with modified deoxyribonuclease, and to Dr. Ta-hsiu Liao for advice on the use of a modified analyzer in the nanomole range. We are indebted to Miss Julieta Villanueva for the amino acid analyses on the nanomole scale performed with the Durrum D-500 analyzer.

REFERENCES

1. Félix, F., and Broillet, N. (1966) Biochim. Biophys. Acta 122, 127–144
2. Hata, T., Hayashi, R., and Doi, E. (1961) Agr. Biol. Chem. 31, 357–367
3. Hayashi, R., Aibara, S., and Hata, T. (1970) Biochim. Biophys. Acta 212, 359–361
4. Hayashi, R., and Hata, T. (1972) Biochim. Biophys. Acta 283, 673–679
5. Doi, E., Ogawa, H., Hayashi, R., and Hata, T. (1966) Symposion on Enzyme Chemistry (in Japanese) 18, 349–351
6. Hayashi, R., Minami, Y., and Hata, T. (1972) Agr. Biol. Chem. 36, 621–629
7. Zuber, H. (1964) Nature 201, 613
8. Tschersche, H., and Kupper, S. (1972) Eur. J. Biochem. 20, 33–36
9. Spergler, R., Heilmann, H.-D., Gramp, E., and Uhlig, H. (1971) Hoppe-Seyler’s Z. Physiol. Chem. 352, 1524–1530
10. Wells, J. R. E. (1965) Biochem. J. 97, 228–235
11. Carey, W. F., and Wells, J. R. E. (1972) J. Biol. Chem. 247, 5573–5579
12. Ichishima, E. (1972) Biochim. Biophys. Acta 256, 274–288
13. Imoto, J. N., and Dure, L. S., III. (1972) J. Biol. Chem. 247, 5034–5040
14. Imoto, J. N., and Dure, L. S., III. (1972) J. Biol. Chem. 247, 5041–5047
15. Visubi, K., Mikola, J., and Enari, T.-M. (1969) Eur. J. Biochem. 7, 193–199
16. Hayashi, R., Oka, Y., and Hata, T. (1960) Agr. Biol. Chem. 33, 196–206
17. Aibara, S., Hayashi, R., and Hata, T. (1971) Agr. Biol. Chem. 35, 658–666
18. Crestfield, A. M., Moore, S., and Stein, W. H. (1963) J. Biol. Chem. 238, 622–627
19. Moore, S. (1968) J. Biol. Chem. 243, 6281–6283
20. Moore, S., and Stein, W. H. (1964) J. Biol. Chem. 211, 907–913
21. Ambler, R. P. (1967) Methods Enzymol. 11, 155–166
22. Moore, S., and Seep, W. H. (1965) Methods Enzymol. 6, 810–831
23. Spackman, D. H., Stein, W. H., and Moore, S. (1958) Anal. Chem. 30, 1190–1206
24. Spackman, D. H. (1963) Fed. Proc. 22, 244
25. Hugli, T. E., and Moore, S. (1972) J. Biol. Chem. 247, 2828–2834
26. Hirs, C. H. W. (1967) Methods Enzymol. 11, 59–62
27. Johansen, P. G., Marshall, R. D., and Neuberger, A. (1960) Biochem. J. 77, 239–247
28. Stark, G. R. (1963) Methods Enzymol. 11, 123–138
29. Stark, G. R., and Smyth, D. G. (1963) J. Biol. Chem. 238, 214–226
30. Salnikow, J., Liao, T.-H., Moore, S., and Stein, W. H. (1973) J. Biol. Chem. 248, 1480–1488
31. Davis, B. J. (1964) Ann. N. Y. Acad. Sci. 121 (2), 404–421
32. Wedek, K., and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412
33. Tsugita, A., and Akabori, S. (1959) J. Biochem. (Tokyo) 46, 695–704
34. Waibel, D., and Christian, W. (1946) Biochem. Z. 310, 384–421
35. Lenney, J. F. (1956) J. Biol. Chem. 221, 919–930
36. Hata, K., and Hata, T. (1972) J. Biol. Chem. 246, 639–638
37. Bromfe, W. W., Sinn, L. G., and Behrens, O. K. (1957) J. Amer. Chem. Soc. 79, 2807–2810
38. Sanger, F., and Tuppy, H. (1951) Biochem. J. 49, 481–490
39. Hirs, C. H. W., Moore, S., and Stein, W. H. (1960) J. Biol. Chem. 235, 633–647
40. Smyth, D. G., Stein, W. H., and Moore, S. (1963) J. Biol. Chem. 238, 227–234
41. Hugli, T. E. (1973) J. Biol. Chem. 248, 1712–1718
42. Price, P. A., Liu, T.-Y., Stein, W. H., and Moore, S. (1969) J. Biol. Chem. 244, 917–922
43. Shaw, D. C., and Wells, J. R. E. (1972) Biochem. J. 123, 229–235
44. Liao, T.-H., Salnikow, J., Moore, S., and Stein, W. H. (1973) J. Biol. Chem. 248, 1480–1489
45. Hermanson, M. A., Kuhn, R. W., Walsh, K. A., Neurath, H., Erikson, N., and Benditt, R. (1972) Biochemistry 11, 2084–2098
Carboxypeptidase from Yeast: LARGE SCALE PREPARATION AND THE APPLICATION TO COOH-TERMINAL ANALYSIS OF PEPTIDES AND PROTEINS

Rikimaru Hayashi, Stanford Moore and William H. Stein

J. Biol. Chem. 1973, 248:2296-2302.

Access the most updated version of this article at http://www.jbc.org/content/248/7/2296

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/248/7/2296.full.html#ref-list-1