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DOES CARBOXYPEPTIDASE Y HAVE INTRINSIC ENDOPEPTIDASE ACTIVITY?

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Summary: Carboxypeptidase Y preparations from baker's yeast have been found to exhibit endopeptidase activity when assayed with oxidized insulin B-chain. Amino acid analysis and peptide isolation studies indicate that a specific internal cleavage occurs between Leu-15 and Tyr-16 in addition to the C-terminal carboxypeptidase activity. Blocking the C-terminal residue of the substrate prevents the exopeptidase activity of the enzyme, but has no effect on the endopeptidase activity. On the other hand, pepstatin A inhibits the endopeptidase but not the exopeptidase activity. These results suggest that the endopeptidase activity is due to the presence of contaminating amounts of yeast proteinase A and indicate that caution should be taken when employing carboxypeptidase Y preparations for sequence studies.

Introduction

Carboxypeptidase Y (EC 3.4.12), a glycoprotein exopeptidase from baker's yeast, has broad specificity toward many carboxy-terminal amino acids including proline and amino acid amides. These characteristics have made it particularly attractive for use in sequence studies of proteins. Homogeneous enzyme has been prepared by ion-exchange chromatography[1,2] or by affinity chromatography[3] and both preparations have been reported to be free of endopeptidase activity. We have recently had occasion to employ carboxypeptidase Y for amino acid sequencing studies and found that it exhibits a specific endopeptidase activity. Similar observations have since been reported by others[4]. This endopeptidase activity appears to be due to contamination by yeast proteinase A. Addition of pepstatin A selectively

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inhibits the endopeptidase activity and allows the use of the carboxypeptidase Y preparations for the purposes intended.

Materials and Methods

Carboxypeptidase Y was obtained from Pierce Chemical Company. Pepstatin A and insulin B-chain, oxidized, were purchased from Sigma Chemical Company, and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluene sulfonate was from Aldrich. Digestion of the insulin B-chain with carboxypeptidase Y was carried out in 2-(N-morpholino)ethane sulfonic acid (MES) buffer (Sigma), 10 mM, pH 6.8, for various periods of time. Polyacrylamide gel electrophoresis was run in the presence of sodium dodecylsulfate (SDS) under standard conditions[5]. Amino acid analyses were performed with a Durrum D-500 instrument according to the manufacturer's instructions. Thin-layer chromatography was carried out on silica gel plates (E. Merck, Darmstadt) using $CHCl_3$: CH_3OH : 30% NH_4OH, 2:2:1, as solvent. Hydrolyses were performed *in vacuo* in constant boiling HCl at 110° for 20 hr. Carbodiimide modification of insulin B-chain was carried out in acetate buffer at pH 4.75, in the presence of 1.0 M taurine[6]. The degree of modification was determined by amino acid analysis. Pepstatin A (1.4 mg), dissolved in 0.2 ml of 5% triethylamine (Pierce Sequenal grade), was diluted to 2.0 ml in 10 mM MES, adjusted to pH 6.8. Carboxypeptidase Y was pre-incubated with pepstatin A for 2 min. at room temperature, (final pepstatin concentration, 50 μM prior to assay).

Results

Two different preparations of commercial carboxypeptidase Y were examined for homogeneity by SDS-gel electrophoresis. The enzyme migrated as a single band corresponding to a molecular weight of about 62,000. Overloading of the gel failed to identify any trace contaminant with higher or lower molecular weight

The specificity of carboxypeptidase Y was examined using oxidized insulin B-chain as substrate. A 1 mg (300 nmole) sample of the peptide was dissolved

TABLE I. Amino Acid Content of Total Digest TLC-Fractions from Oxidized Insulin B-Chain Digested with Carboxypeptidase Y

	Total Digest[a]	TLC-Fraction No.[b] 1	2	3	4	5	6[c]	7[c]	8	9	10	Sum[d]	B-Chain[e]
$CySO_3$							1.0	1.0				2.0	2
Asp								1.0				1.0	1
Thr	1.0					+						1.0	1
Ser								0.8				0.8	1
Glu	1.0	+					1.1	0.9				3.0	3
Pro	1.0		+									1.0	1
Gly							1.7	1.0				2.7	3
Ala	1.9			+								1.9	2
Val	1.0						1.0	1.1		+		3.1	3
Leu	1.1						1.1	2.0			+	4.2	4
Tyr	1.0						1.0		+			2.0	2
Phe	1.1						1.0	1.0			+	3.1	3
His								1.8				1.8	2
Lys	1.0	+										1.0	1
Arg							0.7					0.7	1

[a] Relative to 1.0 Thr, before acid hydrolysis.

[b] The + symbols indicate that this amino acid(s) was present in this fraction before and after acid hydrolysis. Quantitation was not attempted.

[c] Relative to 1.0 $CySO_3$, after acid hydrolysis.

[d] The sum of residues in the total digest plus fractions 6 and 7.

[e] The amino acid composition of insulin B-chain.

in 0.4 ml 10 mM MES, pH 6.8, and 10 µl of enzyme solution (0.5 mg/ml in water) was added. Digestion was allowed to proceed for 10 min. at 37°. An aliquot of the reaction mixture was then diluted in citrate buffer, pH 2.2, and injected directly into the D-500 for amino acid analysis (Table I). The remainder of the digest was frozen immediately and lyophilized. Subsequent analysis by thin-layer chromatography, TLC, (Figure 1) indicated the presence of ten fractions, of which eight could be identified as containing free amino

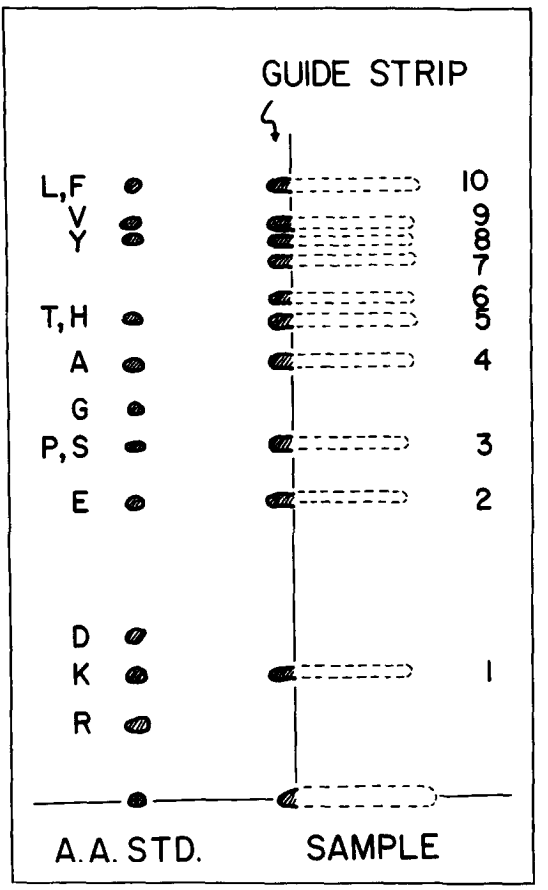

Figure 1. A drawing of the thin-layer chromatogram of the carboxypeptidase Y digest of insulin B-chain. A standard mixture of amino acids was spotted on the left, the digest was streaked at the right. The horizontal line at the bottom corresponds to the origin. The chromatogram was developed with chloroform: methanol: 30% ammonia (2:2:1). After drying, the right side of the chromatogram was covered with a mylar sheet and the left side sprayed with ninhydrin. The areas enclosed by the dashed lines were scraped off, eluted first with 0.5 ml 0.5% acetic acid at 37° for 4 hr, then 0.5 ml 0.5% pyridine at 37° overnight and finally 0.5 ml water. The eluates were combined, evaporated to dryness, and subjected to amino acid analysis before and after hydrolysis with 6N HCl at 110° for 20 hr.

acids (spots 1-5, 8-10, Table I). The two non-amino acid spots were eluted from the plate and subjected to amino acid analysis after acid hydrolysis.

The composition of spot 6 (Table I) was found to correspond to the sequence of insulin B-chain from residue 16 to 24 while that of spot 7 was consistent with the amino terminal segment of the peptide, i.e., residues 1

to 11. The sum of free amino acids released by carboxypeptidase Y digestion and amino acids present in spots 6 and 7 corresponds to the composition of intact insulin B-chain. Essentially identical results were obtained with both samples of carboxypeptidase Y examined.

The presence of two oligopeptides in the insulin B-chain digest suggested the possibility of endopeptidase activity in these carboxypeptidase Y preparations. Such activity was tested by modifying the carboxyl groups of insulin with taurine (2-aminoethylsulfonic acid) via the carbodiimide reaction[6]. Modification was carried out with 11.5 mg oxidized insulin B-chain dissolved in 0.9 ml 0.2 M sodium acetate, 7.5 M urea, pH 4.75, containing 1.0 M taurine. A total of 0.17 g of 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluene sulfonate in 0.3 ml of the acetate-urea buffer was added and reaction was allowed to proceed at room temperature for 24 hr. The pH was maintained at 4.75 using a pH-stat. The reaction mixture was fractionated on a 3 x 50 cm column of BioGel-P2 using water as the eluent. Amino acid analysis indicated the presence of three residues of taurine per insulin B-peptide consistent with the modification of the γ-carboxyl groups of Glu-13 and Glu-21 and the α-carboxyl group of Ala-30.

A sample of the modified B chain (40 μg,12 nmole) was exposed to carboxypeptidase Y (0.8 μg, 12 pmole) in 10 mM MES, pH 6.8, for various periods of time at 37°. Aliquots (1/3 of the total volume) were removed at 5, 10, and 30 min., frozen and lyophilized. The amino acids released were determined by amino acid analysis (Table II). In contrast to the results obtained with B-chain that had not been reacted with taurine-carbodiimide, only Leu, Ala and an acidic residue (identified as γ-tauryl-glutamate) were found. These results are consistent with the blocking of the C-terminal carboxyl group and, further, indicate that cleavage of the insulin B-chain must occur between residues 15 and 16, thereby exposing a new C-terminal residue, Leu-15.

The endopeptidase activity can be abolished by addition of pepstatin A, a potent inhibitor of acidic proteases[7]. Carboxypeptidase Y (5 μg/μl) was

TABLE II. Release of Amino Acids from Taurine-Substituted Insulin B-Chain by Digestion with Carboxypeptidase Y.

Digestion Time (min)	Amino Acid Released (nmole) Leu	Ala	γ-Taur-Glu
5	0.9	0.3	0.3
10	1.8	0.6	0.7
30	3.1	1.2	0.8

incubated with 10 μl of 1 mM pepstatin A in 10 mM MES, pH 6.8 at room temperature for 2 minutes. To this solution was added 100 nmole of oxidized insulin B-chain (200 μl in 10 mM MES, pH 6.8). Digestion was allowed to proceed at 37° for 10 minutes before stopping the reaction by rapid freezing and lyophilization. Six amino acids were released as detected by amino acid analysis: Thr(1), Pro(1), Ala(1), Tyr(1), Phe(1) and Lys(1). These correspond to the carboxy-terminal segment from residues 25 to 30 of insulin B-chain. Thus, in the presence of pepstatin A there is no cleavage between residues 15 and 16.

Discussion

Carboxypeptidase Y has been shown to be a broad specificity exopeptidase and has been suggested as a useful enzyme for carrying out protein sequence determinations[2,8]. Our initial results suggested that it might also exhibit a very selective endopeptidase activity. The amino acids released on digestion of oxidized insulin B-chain with carboxypeptidase Y (Table I) are not consistent with the continual progressive action of an exopeptidase. The presence of Val and Leu and the absence of $CySO_3$, Gly and Arg, for example, could only result if sequential hydrolysis ceased prior to the release of Gly-23 and began again after $CySO_3$-19. The exact site of endopeptidase action was determined by examining the amino acid compositions of the two peptides isolated from the digestion mixture. The peptide from spot 6 was shown to contain residues 16-24, and that in spot 7 was shown to contain residues 1-11.

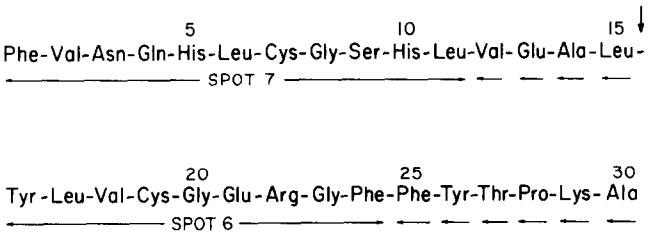

Figure 2. Digestion of insulin B-chain with carboxypeptidase Y. The horizontal arrows (←) indicate residues removed by the action of carboxypeptidase Y. The vertical arrow indicates the peptide bond cleaved by endoprotease activity. The double headed arrows (↔) indicate the peptides isolated by TLC after digestion of B-chain with carboxypeptidase Y.

Residues 12-15 and 25-30 could be accounted for as free amino acids. Thus, as indicated in Figure 2, the carboxypeptidase Y preparations examined remove residues 25-30, sequentially, from the carboxy-terminus. In addition, they cleave the internal peptide bond between Leu-15 and Tyr-16, thereby releasing the nonapeptide 16-24 and exposing a new carboxy-terminus, thus allowing the concomitant sequential hydrolysis of residues 12-15.

Blocking the carboxy-terminal α-carboxyl group of the insulin B-chain with taurine via the carbodiimide reaction prevents the liberation of residues 25-30 (Table II). However, cleavage between residues 15 and 16 still occurs because Leu, Ala and an acidic residue are released on exposure to carboxypeptidase Y. The acidic residue is converted to glutamate and taurine by acid hydrolysis, and is obviously the diimide coupling product of the γ-carboxyl group of Glu-13 and taurine. The point of cleavage, between Leu-15 and Tyr-16, is apparently quite selective. Most chymotrypsin-like enzymes might be expected to cleave the bond between Tyr-16 and Leu-17[9].

The enzyme preparations examined were devoid of contaminating proteins detectable by SDS-gel electrophoresis. However, yeast proteinase A, having a molecular weight, isoelectric point and sugar content similar to that of carboxypeptidase Y[10], could easily be a contaminant. Pepstatin A is a specific inhibitor of acid proteases[7] including yeast proteinase A (P. Bünning,

Table 2. Changes of protein-SSG mixed disulfide (GSS-protein) concentration in the particulate fraction on pretreatment with GSSG and subsequent addition of DTT

Incubation 1st.	2nd.	Total GSH[a]	Free GSH[a]	Free GSSG[b]	GSS-protein[a]
None	None	3.0 ± 0.2	0.1 ± 0.1	N.D.[c]	2.9 ± 0.2
GSSG	None	6.3 ± 0.2	0.6 ± 0.1	0.3 ± 0.1	5.2 ± 0.3**
GSSG	DTT	4.6 ± 0.1	1.0 ± 0.1	0.1 ± 0.1	3.5 ± 0.2

Experimental conditions were as for Table 1. After incubation with or without DTT, the suspension was centrifuged and washed once. The resulting precipitate were assayed for GSH and GSSG. Each value represents the mean ± S.E. of 4 separate experiments. a; nmol GSH/mg protein, b; nmol GSSG/mg protein, c; Not detected. ** $P<0.01$; Compared with "None-None".

The amount of GSS-protein in the enzyme preparation was determined (Table 2). The amount was significantly increased by incubating the preparation with 2.5 mM GSSG ($P<0.01$), and the levels of GSS-protein returned to the control range on further incubation with DTT.

DISCUSSION

Several sulfhydryl reagents inhibit adenylate cyclase from various sources (2-9). Most previous studies have been made with sulfhydryl blocking reagents and have indicated the requirement for free thiol groups for adenylate cyclase activity. In the present study, we found that GSSG but not GSH inactivated adenylate cyclase in rat brain caudate nucleus. The involvement of disulfide exchange as a possible regulatory mechansim for sulfhydryl enzymes has been extensively studied with rabbit liver fructose diphosphatase (10). The idea that a disulfide exchange also occurs during inactivation of adenylate cyclase by GSSG is supported by the present finding that when adenylate cyclase has been inactivated by GSSG it can be almost fully re-