Biochimica et Biophysica Acta, 567 (1979) 503—510

BBA 68702

CHEMICAL AND PHYSICAL PROPERTIES OF THE CARBOXYPEPTIDASE Y-INHIBITOR FROM BAKER'S YEAST *

HEIDRUN MATERN, RENATE BARTH and HELMUT HOLZER **

Biochemisches Institut der Universität Freiburg im Breisgau and Institut für Toxikologie und Biochemie der Gesellschaft für Strahlen- und Umweltforschung, Hermann-Herder-Strasse 7, D-7800 Freiburg im Breisgau (F.R.G.)

(Received September 15th, 1978)

Key words: Carboxypeptidase Y-inhibitor; (Properties, Baker's yeast)

Summary

The purification as well as some characteristics of the carboxypeptidase Y-inhibitor from baker's yeast have been described in a previous report (Matern, H., Hoffmann, M. and Holzer, H. (1974) Proc. Natl. Acad. Sci. U.S. 71, 4874—4878). In this paper, chemical and physical properties of the purified inhibitor are presented. The molecular weight was estimated at 23 400—24 000 and appears to be a monomeric unit. Amino acid analysis and carbohydrate studies are given, showing the existence of three disulfide bonds and one sulfhydryl group per molecule and the absence of carbohydrate residues. The N-terminal amino acid is blocked by an acetyl group. The C-terminal amino acid is lysine. The isoelectric point (p*I*) is 6.6 and the inhibitor-enzyme complex is stable (at 25°C) between pH 5 and 9. The apparent K_i value was calculated as $2.5 \cdot 10^{-9}$ M.

Introduction

The existence of a carboxypeptidase Y-inhibitor in crude yeast extract was first mentioned by Lenney and Dalbec [1] and some properties were described from a partially purified material [2]. A carboxypeptidase Y-inhibitor complex was isolated by Hayashi et al. [3], but the native inhibitor could not be

* Parts of this publication have been presented as a preliminary account at the Frühjahrstagung der Gesellschaft für Biologische Chemie in Heidelberg [7].

** To whom correspondence should be addressed.

Abbreviations: Ac-Tyr-O-Et, *N*-acetyl-L-tyrosine ethyl ester; dansyl, 5-dimethylaminophthalene-1-sulfonyl; SDS, sodium dodecyl sulfate.

dissociated from the complex [4,5]. In a previous paper from this laboratory [6], the separation of the inhibitor from the complex and its isolation and partial characterization were reported. In this communication, chemical and physical properties of the inhibitor are described.

Materials and Methods

An amino acid calibration mixture was obtained from Beckman (Munich, F.R.G.). The reagents for the enzymic determination of acetate were purchased from Boehringer (Mannheim, F.R.G.). Guanidine-HCl for spectroscopic purpose, iodoacetamide, phenylmethylsulfonyl fluoride, *p*-mercuribenzoate and carboxypeptidase A from bovine pancreas (treated with diisopropyl phosphorofluoridate) were from Serva (Heidelberg, F.R.G.). Carboxypeptidase B (treated with phenylmethylsulfonyl fluoride) was obtained from E. Merck (Darmstadt, F.R.G.). Ellman's reagent was from Fluka (Buchs, Switzerland). Carboxypeptidase Y was purchased from the Oriental Yeast Company (Tokyo, Japan). The purification of the carboxypeptidase Y-inhibitor from baker's yeast and the source of baker's yeast were the same as previously described [6].

Enzyme and inhibitor assays. Carboxypeptidase Y and carboxypeptidase Y-inhibitor were routinely assayed with Ac-Tyr-O-Et as substrate as previously published [6]. At pH values different from 8.0 carboxypeptidase Y and carboxypeptidase Y-inhibitor were measured by the pH-stat method of Hata et al. [8] with 10 mM Ac-Tyr-O-Et as substrate using the pH-stat assembly ABU 12/TTT 11/PHM 26/SBR 2/TT 31 (Radiometer, Copenhagen, Denmark).

Aminopeptidases I and II [9] and carboxypeptidase C [10] were assayed according to published procedures. Carboxypeptidase S activity was determined as described by Wolf and Weiser [11], with *N*-benzyloxycarbonyl-Gly-Leu as substrate.

Electrophoretic procedures. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was performed according to the method of Mason et al. [12] in 12% polyacrylamide gels.

Gel electrofocusing was carried out as described by Wrigley [13]. 7.5% polyacrylamide gels containing 1% carrier ampholytes (pH 5—8) were used. After electrofocusing at 400 V for 4 h, each gel was cut into pieces and these pieces were minced and extracted with 0.5 ml water; pH and inhibitory activity of the extracts were measured.

Amino acid analysis and end group determination. Amino acid analysis was performed with a Biotronik amino acid analyzer LC 6000. Desalted protein samples (200 μg each) were hydrolyzed with 6 N HCl at 105 ± 0.5°C for 24, 48, 72 and 96 h in sealed, evacuated ampoules. The hydrolysates were analyzed according to the method of Spackman et al. [14] by separation with a single column [15]. Cysteic acid and methionine sulfone were determined after performic acid oxidation, according to Moore [16]. Tryptophan was estimated spectrophotometrically in 20 mM potassium phosphate (pH 6.5)/6 M guanidine-hydrochloride, as described by Edelhoch [17].

For determination of acetyl as the blocking group of the N-terminal amino acid, 30 nmol inhibitor were hydrolyzed with 6 N HCl at 105 ± 0.5°C for 5 h in sealed, evacuated ampoules, followed by extraction of acetic acid as

described by Kuo and Younathan [18]. Acetate was estimated by a series of enzyme reactions, resulting in an acetate-dependent oxidation of NADH, catalyzed by acetyl-CoA synthetase, myokinase, pyruvate kinase and lactate dehydrogenase (Möllering, H., unpublished results).

The C-terminal amino acid was identified according to Ambler [19]. Digestions were performed with carboxypeptidase A from bovine pancreas at pH 9.2, using an enzyme/substrate ratio of 1 : 50 (mol/mol) and with carboxypeptidase B from bovine pancreas at pH 8.5 using and enzyme/substrate ratio of 1 : 260 (mol/mol). The supernatants after ethanol precipitation (85%, v/v) and enzyme digestion, were analyzed for free amino acids by thin-layer chromatography as described by Crowshaw et al. [20] and by amino acid analysis.

Other analytical methods. Sedimentation-diffusion equilibrium studies for molecular weight determination were performed in a Beckman model E ultracentrifuge at 4°C and 60 000 rev./min in a 6-channel rotor according to Yphantis [21]. The protein absorption was recorded with a photoelectric scanner.

Carbohydrate analysis of the inhibitor was performed using the periodic acid-Schiff staining procedure for glycoproteins as described by Segrest and Jackson [22]. The inhibitor was subjected to polyacrylamide gel electrophoresis according to the method of Davis [23] with 7.5% polyacrylamide gels. The gels were stained for glycoprotein, scanned at 534 nm with a Gilford spectrophotometer No. 250, and the amount of carbohydrate was estimated by planimetry using a calibration curve obtained with carboxypeptidase Y as glycoprotein standard.

Protein concentrations were determined by the method of Lowry et al. [24] with crystalline bovine serum albumin as standard.

Results and Discussion

Molecular weight

Sedimentation-diffusion equilibrium studies of the inhibitor in the analytical ultracentrifuge indicated homogeneity of the sample, and a molecular weight of 24 000 was calculated [21]. This value is based on a partial specific volume of 0.72 ml/g, which was obtained from the amino acid composition (see Table I), according to Cohn and Edsall [25].

From amino acid analysis, the molecular weight was calculated to be 23 400, on the basis of 3 arginine residues per molecule protein.

SDS-polyacrylamide gel electrophoresis yielded a molecular weight of 28 000 ± 15%. The approximate coincidence between the molecular weights obtained by ultracentrifugation, gel filtration [6] and by SDS-polyacrylamide gel electrophoresis indicates that the inhibitor has no subunit structure.

A previous calculation of Hayashi and Hata [5], using the difference in molecular weight of the carboxypeptidase Y-inhibitor complex and carboxypeptidase Y, gave a molecular weight of about 19 000 for the inhibitor. As these authors prepared the carboxypeptidase Y-inhibitor complex from autolyzed yeast, the 5000 dalton less compared to the isolated inhibitor might be due to proteolytic attack during autolysis.

TABLE I

AMINO ACID COMPOSITION OF CARBOXYPEPTIDASE Y-INHIBITOR FROM BAKER'S YEAST

Amino acid	Residues per molecule				
	Hydrolysis (h)				Average or extrapolated integer
	24	48	72	96	
Asp	23.2	21.7	22.0	22.3	22
Thr	13.1	12.8	12.6	12.3	14 [a]
Ser	18.4	17.6	15.8	14.7	20 [a]
Glu	22.3	21.6	21.7	21.5	22
Pro	14.2	14.4	13.6	13.6	14
Gly	13.1	13.0	12.4	12.6	13
Ala	16.5	16.3	16.5	16.2	16
Cys [c]	7.4	—	—	—	7
Val	9.1	9.7	10.1	10.4	10 [b]
Met [c]	5.6	5.3	5.4	5.5	5
Ile	4.5	5.2	5.4	5.8	6 [b]
Leu	12.1	12.2	12.4	12.3	12
Tyr	6.3	6.2	6.5	6.4	6
Phe	12.5	12.3	12.3	12.1	12
Lys	18.4	18.3	18.6	19.0	19
His	5.1	5.4	5.2	5.2	5
Arg	3.0	3.0	3.0	3.0	3
Trp [d]	—	—	—	—	3

[a] Extrapolated to zero hydrolysis time.
[b] Rounded off value of 96 h hydrolysis time.
[c] Determined by performic acid oxidation [16].
[d] Determined by spectral analysis [17].

Amino acid analysis

The amino acid analysis of the inhibitor (Table I) gives a polypeptide with 209 residues. The 20 common amino acids are present in the carboxypeptidase Y-inhibitor in contrast to the proteinase A-inhibitor from yeast which lacks cysteine, tryptophan, arginine and proline [26] and the proteinase B-inhibitor from yeast, which lacks cysteine, tryptophan, arginine and methionine [26].

Determination of free sulfhydryl groups, with the Ellman reagent [27] in the presence of 8 M urea, yielded 1.3 thiol residues per molecule protein. Therefore, the 7 half-cystine residues per molecule inhibitor constitute three disulfide bridges and one sulfhydryl group. Most of the investigated proteinase inhibitors contain a large number of disulfide bridges, which in many cases have been shown to locate the reactive center within a polypeptide loop [28].

Corresponding to the presence of 6 tyrosine and 3 tryptophan residues per molecule protein the ultraviolet absorption spectrum shows a maximum at 276 nm and a shoulder near 290 nm in 20 mM potasssium phosphate (pH 6.5)/6 M guanidine-HCl.

End groups

Experiments to identify the N-terminal amino acid of the inhibitor failed, suggesting that the N-terminal amino group was blocked. Treatment with dansyl chloride according to Hartley [29], followed by acid hydrolysis and separation of the dansylated amino acids by thin-layer chromatography [29]

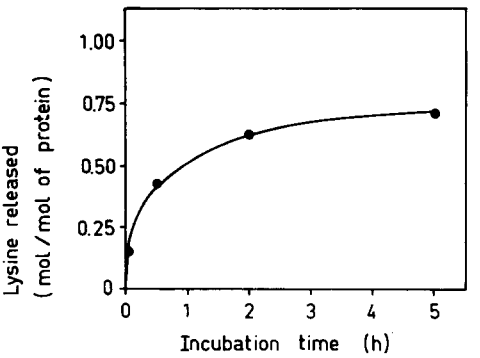

Fig. 1. Rate of release of lysine from the inhibitor by digestion with carboxypeptidase B from bovine pancreas in 0.2 M *N*-ethylmorpholine acetate, pH 8.5, at 25°C. Samples taken after different times of incubation were analyzed for amino acids.

and, alternatively, Edman degradation with phenylisothiocyanate [30] failed to reveal an N-terminal amino acid. Since N-terminal amino acids are most frequently blocked by an acetyl group [31], determination of acetyl as blocking group was performed. 30 nmol inhibitor gave 17 nmol acetate. The amount of acetate that could be determined in parallel experiments using the acetylated proteins alcohol dehydrogenase from horse liver [32] and ovalbumin [33] was about 50% of the theoretical value. Bovine serum albumin, which is not acetylated [34], did not yield acetate in these experiments. From these results, we propose that the N-terminal of the carboxypeptidase Y-inhibitor is blocked by an acetyl group.

For determination of the C-terminal amino acid, digestions of the inhibitor were performed with carboxypeptidases A and B from bovine pancreas. As shown in Fig. 1, incubation of the inhibitor with carboxypeptidase B at pH 8.5 led to a time-dependent release of lysine as the only amino acid. With carboxypeptidase A also, lysine as the only hydrolysis product could be detected when the pH was raised to 9.2 according to Davie et al. [35], who reported that hydrolysis of basic amino acids by carboxypeptidase A can be accelerated by an increase in pH. From these results, it is concluded that lysine is the C-terminal amino acid.

Other chemical and physical properties

From determinations of the inhibitory activity and pH values in extracted gel slices obtained after isoelectric focusing of the inhibitor in polyacrylamide gel an p*I* of 6.6 was calculated. When stained with amido black, the inhibitor exhibited one single band, which appeared at pH 6.6.

Measurement of carbohydrate with the periodic acid-Schiff staining procedure for glycoproteins [22] showed that the inhibitor contains less than 0.2% carbohydrate. This corresponds to less than 0.25 mol hexose per mol inhibitor.

Protein determinations according to Lowry et al. [24] were in good agreement with values calculated from amino acid analysis. Thus, in accordance with ultraviolet absorption, there is no indication that the inhibitor contains compounds other than amino acids.

Inhibitory properties

Sulfhydryl reagents such as 1 mM *p*-mercuribenzoate, 10 mM iodoacetamide or the Ellman reagent, whose reaction with the free SH-group can be demonstrated spectroscopically [27], did not affect the activity of the inhibitor against carboxypeptidase Y, whereas carboxypeptidase Y was inhibited by these reagents [36,37]. The free sulfhydryl group is, therefore, not required for inhibitory activity. 1 mM phenylmethylsulfonyl fluoride, which is an inhibitor of carboxypeptidase Y by acting on serine at the active site [37], had no effect on the activity of the inhibitor, suggesting that serine is not part of the inhibitory site.

With Ac-Tyr-O-Et as substrate, the inhibitory activity against carboxypeptidase Y was about the same between pH 5 and 9. Maximum Ac-Tyr-O-Et hydrolyzing activity of carboxypeptidase Y was obtained at pH 8.0 which is in agreement with the findings of Doi et al. [36].

Inhibition of the Ac-Tyr-O-Et hydrolyzing activity of carboxypeptidase Y at pH 8.0 caused by increasing levels of the inhibitors is shown in Fig. 2. The titration curve was linear up to 80% inhibition of carboxypeptidase Y; 100% inhibition was obtained. From these data the apparent K_i value ($1/K_{assoc}$) was calculated graphically to be $2.5 \cdot 10^{-9}$ M as described for nonstoichiometric inhibition by Bieth [38].

In a previous paper from this laboratory [6], some results concerning the proteinase specificity of the inhibitor have been presented. The inhibitor did not inhibit yeast proteinases A and B or bovine pancreas carboxypeptidases A and B. The high specificity of the inhibitor for carboxypeptidase Y was further substantiated by showing that the inhibitor did not inhibit the aminopeptidases I and II [9] and carboxypeptidase S [11] from yeast or carboxypeptidase C [10] from citrus fruits.

The high proteinase specificity of the carboxypeptidase Y-inhibitor suggests a specific function of the inhibitor in the control of carboxypeptide Y activity in yeast. Together with the other known proteinase inhibitors from yeast, the proteinase A- and B-inhibitors [39—41], the carboxypeptidase Y-inhibitor is

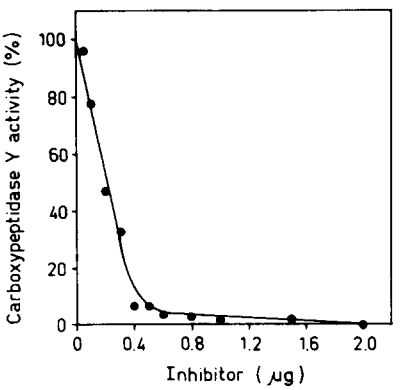

Fig. 2. Inhibition of carboxypeptidase Y (0.8 μg) by increasing amounts of the inhibitor. Carboxypeptidase Y activity was assayed with Ac-Tyr-O-Et as substrate [6].

localized in the cytosol [42,43] whereas the respective proteinases are found exclusively in the vacuoles of the yeast cell [44]. It may, therefore, be assumed that besides their role in the regulation of proteolytic processes a main biological function of the inhibitors is the protection of extravacuolar space proteins against unwanted proteolytic damages which might be caused by proteinases released from leaky or broken vacuoles [41,43,45,46]. The results on the chemical and physical properties of the carboxypeptidase Y-inhibitor presented in this communication should serve as a basis for further studies concerning the biological function of the inhibitor.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 46, and the Verband der Chemischen Industrie e.V. (Fonds der Chemischen Industrie). The authors are indebted to Professor Dr. H. Zuber for a gift of carboxypeptidase C, Dr. P. Bünning and Dr. D.H. Wolf for samples of partially purified preparations of aminopeptidases I and II and carboxypeptidase S, Dr. R. Tesch for the automated Edman degradation and Dr. E.-G. Afting and Chem. Ing. H.P. Henninger for the analytical ultracentrifugation. The excellent assistance of Mrs. D. Montfort and Mrs. L. Kötschau in the preparation of the manuscript is gratefully aknowledged.

References

1 Lenney, J.F. and Dalbec, J.M. (1969) Arch. Biochem. Biophys. 129, 407—409
2 Lenney, J.F. (1975) J. Bacteriol. 122, 1265—1273
3 Hayashi, R., Oka, Y. and Hata, T. (1969) Agric. Biol. Chem. 33, 196—206
4 Hayashi, R., Minami, Y. and Hata, T. (1972) Agric. Biol. Chem. 36, 621—629
5 Hayashi, R. and Hata, T. (1972) Agric. Biol. Chem. 36, 630—638
6 Matern, H., Hoffmann, M. and Holzer, H. (1974) Proc. Natl. Acad. Sci. U.S. 71, 4874—4878
7 Matern, H. and Holzer, H. (1975) Hoppe-Seyler's Z. Physiol. Chem. 356, 253
8 Hata, T., Hayashi, R. and Doi, E. (1967) Agric. Biol. Chem. 31, 357—367
9 Masuda, T., Hayashi, R. and Hata, T. (1975) Agric. Biol. Chem. 39, 499—505
10 Zuber, H. (1968) Hoppe-Seyler's Z. Physiol. Chem. 349, 1337—1352
11 Wolf, D.H. and Weiser, U. (1977) Eur. J. Biochem. 73, 553—556
12 Mason, T.L., Poyton, R.O., Wharton, D.C. and Schatz, G. (1973) J. Biol. Chem. 248, 1346—1354
13 Wrigley, C.W. (1971) Methods Enzymol. 22, 559—564
14 Spackman, D.H., Stein, W.H. and Moore, S. (1958) Anal. Chem. 30, 1190—1206
15 Hamilton, P.B. (1963) Anal.Chem. 35, 2055—2064
16 Moore, S. (1963) J. Biol. Chem. 238, 235—237
17 Edelhoch, H. (1967) Biochemistry 6, 1948—1954
18 Kuo, S.C. and Younathan, E.S. (1973) Anal. Biochem. 55, 1—8
19 Ambler, R.P. (1972) Methods Enzymol. 25, 143—154
20 Crowshaw, K., Jessup, S.J. and Ramwell, P.W. (1967) Biochem. J. 103, 79—85
21 Yphantis, D.A. (1964) Biochemistry 3, 297—317
22 Segrest, J.P. and Jackson, R.L. (1972) Methods Enzymol. 28, 54—63
23 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
24 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
25 Cohn, E.J. and Edsall, J.T. (1943) Proteins, Amino Acids and Peptides as Ions and Dipolar Ions, pp 370—375, Reinhold Publ. Corp., New York, NY
26 Holzer, H., Bünning, P. and Meussdoerffer, F. (1977) in Acid Proteases (Tang, J., ed.), pp. 271—289 Plenum Publ. Corp., New York, NY
27 Ellman, G.L. (1959) Arch. Biochem. Biophys. 82, 70—77
28 Ozawa, K. and Laskowski, M., Jr. (1966) J. Biol. Chem. 241, 3955—3961
29 Hartley, B.S. (1970) Biochem. J. 119, 805—822
30 Edman, P. (1949) Arch. Biochem. 22, 475—476

31 Needleman, S.B. (1975) Protein Sequence Determination, 2nd edn., pp. 95—103, Springer-Verlag Berlin
32 Jörnvall, H. (1970) Eur. J. Biochem. 14, 521—534
33 Narita, K. and Ishii, J. (1962) J. Biochem. 52, 367—373
34 Shearer, W.T., Bradshaw, R.A. and Gurd, F.R.N. (1967) J. Biol. Chem. 242, 5451—5459
35 Davie, E.W., Newman, C.R. and Wilcox, P.E. (1959) J. Biol. Chem. 234, 2635—2641
36 Doi, E., Hayashi, R. and Hata, T. (1967) Agric. Biol. Chem. 31, 160—169
37 Kuhn, R.W., Kenneth, A.W. and Neurath, H. (1974) Biochemistry 13, 3871—3877
38 Bieth, J. (1974) in Bayer-Symposium V, Proteinase Inhibitors (Fritz, H., Tschesche, H., Greene, L.J and Truscheit, E., eds.), pp. 463—469, Springer-Verlag, Berlin
39 Saheki, T., Matsuda, Y. and Holzer, H. (1974) Eur. J. Biochem. 47, 325—332
40 Núñez de Castro, I. and Holzer, H. (1976) Hoppe-Seyler's Z. Physiol. Chem. 357, 727—734
41 Betz, H., Hinze, H. and Holzer, H. (1974) J. Biol. Chem. 249, 4515—4521
42 Matern, H., Betz, H. and Holzer, H. (1974) Biochem. Biophys. Res. Commun. 60, 1051—1057
43 Lenney, J.F., Matile, Ph., Wiemken, A., Schellenberg, M. and Meyer, J. (1974) Biochem. Biophys Res. Commun. 60, 1378—1383
44 Matile, Ph. and Wiemken, A. (1967) Arch. Mikrobiol. 56, 148—155
45 Holzer, H., Betz, H. and Ebner, E. (1975) in Current Topics in Cellular Regulation (Horecker, B.L and Stadtman, E.R., eds.), Vol. 9, pp. 103—156, Academic Press, New York, NY
46 Holzer, H. (1976) in Metabolic Interconversion of Enzymes 1975 (Shaltiel, S., ed.), pp. 168—174 Springer-Verlag, Berlin