Biochimica et Biophysica Acta, 524 (1978) 102—108

BBA 68434

AMINO ACID SEQUENCE AT THE ACTIVE SITE OF β-GLUCOSIDASE A FROM BITTER ALMONDS

GÜNTER LEGLER and ACHIM HARDER
Institut für Biochemie der Universität Köln, Zülpicher Str. 47, D-5000 Köln 1 (G.F.R.)
(Received September 30th, 1977)

Summary

β-Glucosidase A from bitter almonds was inhibited by the substrate analogue 6-bromo-3,4,5-trihydroxycyclo[2-^{3}H]hex-1-ene oxide. Incorporation of 2 mol inhibitor/mol of dimeric enzyme resulted in total loss of activity. From tryptic digests of the labeled enzyme two radioactive peptides were isolated and their sequence determined (binding site of inhibitor underlined): peptide I, containing approx. 60% of the label: Ile-Thr-Glx-Glx-Gly-Val-Phe-Gly-Asp-Ser-Glx-(Ala, Asx_2, Pro)-Lys and peptide II with approx. 30% of the label: Gly-Thr-Glx-Asp.

The specifity of the reaction of β-glucosidases (β-D-glucoside glucohydrolase, EC 3.2.1.21) with substrate-related epoxides indicates that the aspartic acid labeled in peptide I participates in the catalytic process of β-glucoside hydrolysis. The labeling of a second site is interpreted in terms of two, mutually exclusive, binding modes of the inhibitor.

Introduction

β-Glucosidases A and B (β-D-glucoside glucohydrolase, EC 3.2.1.21) from sweet almonds have been irreversibly inhibited by the active-site-directed inhibitor 6-bromo-3,4,5-trihydroxycyclohex-1-ene oxide (6-bromo-6-deoxy-conduritol B epoxide) with stoichiometric binding of the inhibitor as an ester. The functional groups involved in protonation of the epoxide and subsequent covalent interaction are thought to be the same groups that are essential for the catalytic process of glucoside hydrolysis [1]. In this paper we report the isolation and amino acid sequence of two labelled peptides from β-glucosidase A from bitter almonds that had been inhibited with the above epoxide.

Experimental

β-Glucosidase activity was determined with 20 mM *p*-nitrophenyl β-D-glucopyranoside in 50 mM sodium acetate/HCl pH 5.0 at 35°C. Specific activity

is expressed as U/mg (μmol substrate hydrolyzed · min^{-1} · mg^{-1} protein).

Protein concentration of the purified enzyme was determined by ultraviolet absorption using $A_{280}^{1\,mg/ml} = 1.88$ [1].

Amino acid analyses were carried out on a Beckman Unichrom amino acid analyser with microcells (single column procedure). Peptides were hydrolyzed with 6 M HCl under nitrogen for 14 h at 110—115°C. Molar composition was calculated from the amount of amino acid found and the radioactivity applied to the analyser. Tryptophan was determined from ultraviolet absorbance at 281 nm with a 40 μM solution of peptide in 10% acetic acid.

β-Glucosidase A was isolated from defatted bitter almond meal (Cesar and Loretz, D-4010 Hilden) as described for the enzymes from sweet almonds [1] with the following alterations: in order to prevent evolution of HCN the almond meal was first extracted with acetone/water (3 : 2, v/v) to remove amygdalin, then with acetone, dried, and processed as in ref. [1] up to the last acetone precipitation. All operations were carried out at 0—4°C. The crude enzyme was chromatographed on CM-cellulose (Whatman CM 32) equilibrated with 5 mM sodium acetate (pH 5.8). β-Glucosidase B and flavoprotein (oxynitrilase) were eluted together by a continuous increase in buffer concentration to 50 mM; β-glucosidase A was eluted with a gradient from 50 to 150 mM of the same buffer. The combined fractions containing β-glucosidase A were concentrated to 5 mg/ml by ultrafiltration. Addition of $(NH_4)_2SO_4$ to 40% saturation precipitated a small amount of protein that was removed by centrifugation. The $(NH_4)_2SO_4$ concentration was slowly increased by evaporation at —10°C. The precipitated enzyme was collected after the concentration of $(NH_4)_2SO_4$ had increased to about 70% saturation. Yield: 85 mg/kg almond meal.

Purity was checked by gel electrophoresis in the presence of SDS as described by Weber and Osborn [2] and by isoelectric focussing (LKB ampholine column type 8101) in a 1 : 1 mixture of ampholine pH 4—6 and pH 6—8 (final ampholine concentration 1.6%).

6-Bromo-3,4,5-trihydroxycyclo[2-^{3}H]hex-1-ene oxide ([^{3}H]epoxide) with a specific activity of $2.86 \cdot 10^6$ cpm/μmol was prepared according to the method of ref. 1.

Labeling of the enzyme. To 80 mg β-glucosidase A in 10 ml 50 mM sodium acetate (pH 5.0) were added 30 μmol [^{3}H]epoxide in 2 ml water and the mixture incubated for 24 h at room temperature. Residual β-glucosidase activity was below 1%. Excess inhibitor was removed by chromatography on Sephadex G-200.

Stability of bound label at high pH. 10 nmol of labeled enzyme were denatured in 100 μl 6 M guanidinium chloride at pH 5. Samples of 3 nmol were pipetted into 2.5 ml 0.3 M phosphate buffer of pH 7, 8 and 9. 200-μl aliquots were removed after various time intervals and pipetted onto 300 mg Dowex 50 W, 200—400 mesh, buffered at pH 4.0. Released label was determined as unbound radioactivity in the supernatant.

Denaturation, reduction, and alkylation of the labeled enzyme. 0.8 μmol (70 mg) of labeled β-glucosidase were denatured in 5 ml 6 M guanidinium chloride at pH 5.0 overnight. 0.6 mmol dithiothreitol were added under N_2 and the acidity adjusted to pH 7.1 with 2 N NaOH. After 3 h 1.5 mmol acrylo-

nitrile were added and, after another 15 min, pH was adjusted to 5.0 with 2 N acetic acid, the mixture dialyzed against water, and lyophilized.

Cleavage with CNBr. 0.7 μmol of denatured and alkylated protein dissolved in 1.4 ml 70% formic acid was treated with 15 mg CNBr for 24 h at room temperature. The material was chromatographed in 10% acetic acid on Sephadex G-75 (column 1.5 × 100 cm). 43% of the radioactivity was eluted in the molecular weight range 20000—30000, 47% appeared in the exclusion volume. The latter material was recovered by lyophilisation and again treated with CNBr, this time with 250 mg in 3 ml 70% formic acid. High molecular weight material from this reaction was again subjected to CNBr-cleavage under the same conditions. Fractions eluted in the 20000—30000-dalton range were combined and freeze-dried. The yield, based on radioactivity, was 71%.

Tryptic hydrolysis of CNBr-peptides. To 0.5 μmol CNBr-peptides (based on radioactivity) dissolved in 10 ml 0.3 M phosphate buffer (pH 7.3) were added 3 mg TosPheCH$_2$Cl-treated trypsin (Merck). After 1 h another 3 mg trypsin were added and incubated for a further 2 h at room temperature. The solution was centrifuged and the supernatant adjusted to pH 5. The sediment was resuspended in buffer pH 7.3, treated with 2 mg trypsin for 2 h and processed as above. The combined supernatants were freeze-dried, dissolved in 1 ml 10% acetic acid and chromatographed on Sephadex G-50 (column 2 × 150 cm) with 5% acetic acid as eluant.

Isolation of labeled peptides. The following steps of consecutive chromatography of the radioactive peaks b and c (Fig. 1) led to homogeneous peptides, as judged from chromatographic behaviour, end-group analysis and amino acid composition.

Peak b:

(1) SP-Sephadex G-25 (column 1 × 36 cm) with a linear gradient pyridine/acetic acid from 50 mM (pH 2.9) to 1 M (pH 5.7) (250 ml each).

(2) Biogel P-6 (column 1.5 × 120 cm) in 1 M acetic acid

Peak c:

(1) SP-Sephadex C-25 as above, linear gradient pyridine/acetic acid from 50 mM (pH 2.9) to 150 mM (pH 3.6)

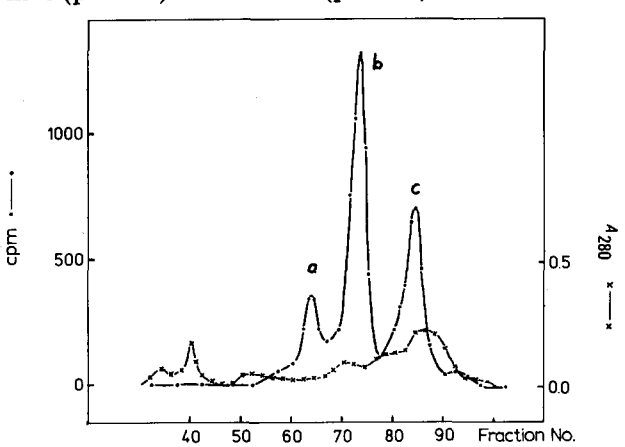


Fig. 1. Chromatography on Sephadex G-50 of tryptic hydrolysate of radioactive BrCN-cleavage products from β-glucosidase A labeled with [^{3}H]epoxide. Column 2 × 150 cm, fraction size 5 ml, ×———×: absorbance at 280 nm; ●———●: cpm in 50 μl eluant.

(2) Biogel P-2 in 1 M acetic acid, (3) Sephadex G-15 in 1 M acetic acid

Peptic hydrolysis of radioactive peptide from peak b. 40 nmol (based on radioactivity) of lyophilized peptide dissolved in 1 ml 30% acetic acid were incubated for 5 h with 0.03 mg pepsin, (twice crystallised, Serva, D-6900 Heidelberg). The reaction was terminated by the addition of 0.2 ml pyridine. Successive chromatography on Sephadex G-25 and SP-Sephadex C-25 (linear gradient from 20% acetic acid to 1 M pyridinium acetate, pH 4.6) gave a pure peptide, as judged by end-group analysis.

Determination of N-terminal amino acids and Edman degradation. This was carried out by the procedures described by Woods and Wang [3] and by Hartley [4]. Radioactivity was determined in the butyl acetate soluble material obtained after each step of the Edman degradation to locate the position of the inhibitor.

Results and Discussion

The β-glucosidase A investigated in this work had the following properties: mol. wt. (gel chromatography): 80000 (at pH 4.6), 155000 (at pH 7.0); (SDS gel electrophoresis) 90000. A similar pH-dependent association-dissociation was observed for bitter almond β-glucosidase A by Sinnott (Sinnott, M.L., unpublished data). The isoelectric point was pH 6.5, the specific activity 105 U/mg.

Heat denaturation in 5 mM acetate (pH 5.8) at 65°C showed a biphasic behaviour in a semilogarithmic plot of activity against time with rate constants of 0.053 min^{-1} and 0.012 min^{-1}. The more stable component appeared to contribute 5—7% of the initial activity. Different proportions of the more stable component were observed at other temperatures. This temperature dependency indicates that different states of a single protein might be the cause of the non-homogeneous activity loss [5].

Stoicheiometry of inhibitor binding

The specific radioactivity of the inhibited enzyme was $3.3 \cdot 10^4$ cpm/mg. This corresponds to an equivalent weight of 86000, in good agreement with the subunit molecular weight found at pH 4.6.

Stability of the enzyme inhibitor bond

While this bond is very stable in the native enzyme [1] it is greatly labilized against alkaline hydrolysis after denaturation. Rate constants for release of

TABLE I

HYDROLYTIC CLEAVAGE OF THE ENZYME-INHIBITOR BOND IN DENATURED β-GLUCOSIDASE A AS FUNCTION OF pH AT 20°C

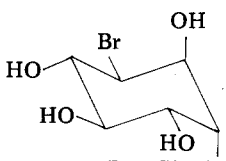
pH	Rate constant (s^{-1})	Half-life (h)
7.0	$1.7 \cdot 10^{-5}$	11.3
8.0	$3 \cdot 10^{-5}$	6.2
9.0	$9 \cdot 10^{-5}$	2.0

label at different pH are given in Table I. A similar lability has been found with conduritol B epoxide bound to β-glucosidase A_3 from *Aspergillus wentii* [6] and to the sucrase-isomaltase complex from rabbit small intestine [7] where an analogous inhibition takes place. The modification of published procedures for reduction, alkylation and tryptic hydrolysis described in the experimental section reduced the losses of label to an insignificant amount. Particularly helpful was the use of a high trypsin-to-substrate ratio at near neutral pH.

The results of chromatography on Sephadex G-50 of the fragments obtained after successive cleavage with cyanogen bromide and trypsin are given in Fig. 1. Peak a (approx. 10% of the total radioactivity) probably represents incompletely degraded material of high molecular weight; it was not investigated further. Peaks b and c (approx. 60% and approx. 30% of the total radioactivity) were purified to homogeneity by ion exchange chromatography and gel chromatography. The radioactivity eluted after peak c represents inhibitor released during the degradation.

Peptide from peak b

The amino acid composition is given in Table II. The Edman degradation could be carried out for ten consecutive steps; thereafter, results became ambigous. Radioactivity appeared in the butyl acetate phase on cleaving off the ninth amino acid (aspartic acid). Results from the Edman degradation and specificity of trypsin given the following structure for this peptide (peptide I):



Ile-Thr-Glx-Glx-Gly-Val-Phe-Gly-Asp-Ser-(Ala, Asx_2, Glx, Pro)-Lys (The inhibitor is bound as an ester [1] to the β-carboxyl group of aspartic acid).

TABLE II

AMINO ACID COMPOSITION OF RADIOACTIVE PEPTIDE FROM PEAK b (FIG. 1)

Other amino acids including tryptophan (from absorbance at 281 nm) were below 0.1 mol/mol.Values are based on radioactivity applied to the analyzer and specific activity of inhibitor.

Amino acid	mol/mol peptide
Aspartic acid	3.1
Threonine	1.1
Serine	0.8
Glutamic acid	3.3
Proline	1.2
Glycine	2.0
Alanine	0.8
Valine	1.1
Isoleucine	0.9
Leucine	0.3
Phenylalanine	0.9
Lysine	1.0

TABLE III

AMINO ACID COMPOSITION OF RADIOACTIVE PEPTIDE FROM PEAK c (FIG. 1)

The amount of other amino acids was below 0.2 mol/mol.

Amino acid	mol/mol peptide
Aspartic acid	1.1
Threonine	0.9
Glutamic acid	0.9
Glycine	1.0

Digestion of peptide I with pepsin permitted the isolation of a radioactive peptide with the compisition Asp, Glx, Gly, Ser, with glycine in the N-terminal position. From this we can conclude that cleavage with pepsin had occurred between phenylalanine and glycine and that the third glutamic acid (or glutamine) follows after serine.

In spite of the presence of one lysine residue peptide I is eluted from the strongly anionic SP-Sephadex with 0.06 M pyridinium acetate (pH 3.1); an indication of its acidic overall character. Most if not all of the Asx and Glx residues will, therefore, be present with free carboxyl side chain.

Peptide from peak c: purification of the radioactive material from peak c resulted in the isolation of a peptide (peptide II) which had the composition (Table III) and amino acid sequence Gly-Thr-Glx-Asp. The radioactivity was bound to aspartic acid. No arginine or lysine could be detected. We therefore have to conclude that peptide II comes from the C-terminal end of the polypeptide chain. Lack of material prevented experiments to confirm the presence of C-terminal aspartic acid with carboxypeptidase or hydrazinolysis. β-Glucosidases A and B from sweet almonds appear to have inaccessible or modified C-terminal amino acids [9].

Amino acids essential to the hydrolysis of glycosidic bonds have been identified by labeling with active site directed epoxides in the following enzymes: egg-white lysozyme [8], β-glucosidase A_3 from Asp. wentii [6], and sucrase-isomaltase from rabbit small intestine [7]. In all cases the inhibitor was bound to an aspartic acid residue; no further similarities, however, are apparent in the published sequences around this residue or in the sequences reported here. It is known from the tertiary structure of lysozyme [10] and there are indications for the Asp. wentii enzyme that amino acids adjacent to the essential aspartic acid are not in contact with the substrate. The dissimilarities are, therefore, not unexpected.

A puzzling result is the appearance of approx. 30% of the label in a second peptide not related to the main fragment. The following reasons may be advanced as an explanation:

(a) Our preparation contains a second β-glucosidase with very similar ion exchange properties, isoelectric point and molecular weight. This should have been detected in the thermal denaturation studies. The enzyme did appear to contain a second, more stable isoenzyme, but only about 7%. Further studies showed that the time course and apparent biphasic denaturation varied with the experimental conditions [11], indicating that other causes are involved.

(b) This enzyme is a hybrid dimer composed of two non-identical subunits. Such hybrids have been reported for β-glucosidases from sweet almonds [12], but in that case equimolar amounts of the two peptides should have been found.

(c) The inhibitor is bound at the active site in more than one mode. The less favorable mode would permit reaction with another carboxyl group, also essential for catalysis. A second carboxyl group participating in the catalytic process has been proposed for lysozyme [10] and for β-glucosidase A_3 from Asp. wentii [13]. Precedents from the literature for a stoichiometric inhibition with inhibitor binding to two mutually exclusive sites are the reaction of His-12 and His-119 with α-halocarboxylates in ribonuclease A [14] and the inhibition of alcohol dehydrogenase from yeast by one mole per subunit of iodoacetamide or iodoacetate which react with two different SH-groups in variable proportions [15].

Acknowledgement

The financial support by the Ministry for Science and Research, Nordrhein-Westfalen, and helpful discussions with Dr. E. Bause are greatfully acknowledged.

References

1 Legler, G. and Hasnain, S.N. (1970) Z. Physiol. Chem. 351, 25—31
2 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
3 Woods, K.R. and Wang, K.T. (1967) Biochim. Biophys. Acta 133, 369—370
4 Hartley, B.S. (1960) Biochem. J. 119, 805—822
5 Laidler, K.J. and Bunting, P.S. (1973) The Chemical Kinetics of Enzyme Action, 2nd edn., pp. 413—460, Clarendon Press, Oxford
6 Bause, E. and Legler, G. (1974) Z. Physiol. Chem. 355, 438—442
7 Quaroni, A. and Semenza, G. (1976) J. Biol. Chem. 251, 3250—3253
8 McKelvy, J.F., Eshdat, Y. and Sharon, N. (1970) Israel J. Chem. 8, 170 p
9 Kleinschmidt, T. and Horst, J. (1971) Z. Physiol. Chem. 352, 603—614
10 Phillips, D.C. (1966) Sci. Am. 215, No. 5, 78—90
11 Becher, H.-J. (1977) Thesis, Univ. Cologne, G.F.R.
12 Kleinschmidt, T. and Horst, J. (1971) Z. Physiol. Chem. 352, 603—614
13 Legler, G. (1968) Z. Physiol. Chem. 349, 767—774
14 Heinrikson, R.L., Stein, W.H., Crestfield, A.M. and Moore, S. (1965) J. Biol. Chem. 240, 2921—2934
15 Belke, C.J., Chin, C.C.Q. and Wold, F. (1974) Biochemistry 13, 3418—3423