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SOME PHYSICOCHEMICAL AND STRUCTURAL PROPERTIES OF TWO β-FUCOSIDASES FROM *ACHATINA BALTEATA*

BERNARD COLAS

Laboratoire de Biochimie, Faculté des Sciences, Université d'Abidjan, Boîte Postale 4322, Abidjan (Republic of the Ivory Coast)

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Summary

Some properties of two β-fucosidases from the digestive juice of a giant snail *Achatina balteata* were studied. Their amino acid composition was similar and both enzymes were shown to be rich in acidic and aromatic residues. Alanine was the single N-terminal residue in the two molecules. The enzymes appeared to be devoid of sialic acid but to contain galactose, glucose, mannose, fucose and hexosamines. The total sugar content was higher in β-fucosidase I (21.6% w/w) than in β-fucosidase II (12.7% w/w).

Gel filtration and ultracentrifugation assays were consistent with an apparent molecular weight higher than 300 000 for β-fucosidase I and of about 110 000 for β-fucosidase II. On SDS polyacrylamide gel electrophoresis, β-fucosidase I dissociated into two subunits (mol. wt. 190 000 and 170 000), whereas β-fucosidase II showed a single band with a mol. w. of about 110 000. On the basis of these studies, it is concluded that the two forms are not structurally related proteins.

Introduction

Data on physicochemical and molecular properties of the β-galactosidases, β-glucosidases and β-fucosidases from animal origin are still limited because these enzymes have been obtained in a less purified state than their microbial homologues [1].

In a previous paper [2], the purification of two β-glycosidases from the digestive juice of a giant African snail *Achatina balteata* has been described. Both are highly specific for the β-anomeric configuration of the glycosidic linkage. They hydrolyse lactose, cellobiose and synthetic β-D-galactosides,

Abbreviation: SDS, sodium dodecyl sulfate.

β-D-glucosides and β-D-fucosides, their most salient property being a very high β-D-fucosidase activity [3]. The purpose of the present report is to investigate the main molecular and physicochemical properties of the two enzymes.

Materials and Methods

Enzymes. β-Fucosidases I and II from the digestive juice of *Achatina balteata*, a land snail found in abundance in West Africa, were purified as described previously [2]. Both were found to be homogeneous by polyacrylamide gel electrophoresis. Their purity was also shown by gel filtration assays on a Sephadex G-200 column. Each enzyme yielded a single peak with a constant specific activity. The specific activity in the fractions was the same as that of the solution charged into the column [3].

Reagents. Chemicals used for polyacrylamide gel electrophoresis were obtained from BDH Chemicals Ltd. Sephadex G-200 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Synthetic substrates: p-nitrophenyl-β-D-galactoside, p-nitrophenyl-β-D-glucoside and p-nitrophenyl-β-D-fucoside were obtained from Sigma Chemical Co., St. Louis, Mo, U.S.A. 1-Dimethylaminonaphtalene-5-sulfonyl chloride (dansyl chloride) and dansyl amino acids were obtained from Mann Research Laboratories. Protein standards for molecular weight determination were obtained from Sigma or Boehringer-Mannheim GmbH, London. Unless otherwise stated, all other chemicals used were of analytical grade.

Enzyme assays. Enzymatic activities with p-nitrophenyl-β-D-glycosides as substrates were determined at 37°C with 5 mM substrate in 100 mM sodium acetate buffer (pH 5.4), as described previously [4]. After adding 2 ml of 100 mM carbonate/bicarbonate buffer (pH 10.8), the released *p*-nitrophenol was determined spectrophotometrically at 420 nm.

Gel filtration experiments with native enzymes. The molecular weight of two β-glycosidases was estimated by Sephadex G-200 gel filtration at room temperature according to the procedure of Andrews [5]. The column (2.6 × 80 cm), equilibrated with a 100 mM sodium acetate buffer (pH 5.4)/100 mM NaCl, was eluted with the same buffer (flow rate, 12 ml/h; 3 ml fractions). Marker proteins were ovalbumin (43 000), bovine serum albumin (68 000), rabbit muscle lactate dehydrogenase (140 000), bovine catalase (232 000), *Escherichia coli* β-galactosidase (540 000). Void volume was determined with Blue Dextran 2000. Proteins and enzymes were detected, respectively, by spectrophotometry at 280 nm and activity determination.

Polyacrylamide gel electrophoresis. Analytical polyacrylamide gel electrophoresis was carried out with 7.5% acrylamide gels at pH 8.4 in a Tris/glycine buffer according to the method of Davis [6]. Protein bands were detected by Amido Black staining. Periodic acid-Schiff staining for glycoproteins was performed according to Zacharius et al. [7].

SDS polyacrylamide gel electrophoresis was realized following the method of Weber and Osborn [8] with 5% acrylamide gels (0.7 × 7 cm). The experiments were carried out at pH 7.2 and room temperature for 6 h in 100 mM phosphate buffer/0.1% SDS with a constant intensity of 8 mA/tube. Gels were stained with 0.25% Coomassie Brilliant Blue for 8 h and destained in methanol/acetic

acid/water (5 : 1 : 5, v/v). The calibration curve was obtained with trypsin, yeast alcohol dehydrogenase, ovalbumin, liver catalase, bovine serum albumin (monomer, dimer and trimer). Before electrophoresis, the protein samples were prepared by boiling for 3 min in a 10 mM sodium phosphate buffer (pH 7.0)/ 1% SDS/1% 2-mercaptoethanol.

Ultracentrifugation study. Sedimentation analyses were carried out with a Spinco model E analytical ultracentrifuge equipped with temperature and speed controls and an ultraviolet scanning system. The protein concentration was in the range 0.3—0.5 mg/ml. Prior to each assay, the enzyme solution was dialysed for 12 h against a 50 mM sodium acetate buffer (pH 5.25)/150 mM NaCl. The results were assumed to be equivalent to values at limiting concentration.

Amino acid analysis. The amino acid composition of the enzymes was determined by the method of Spackman et al. [9], using norleucine as internal standard. Hydrolyses were carried out for 12, 24 and 36 h at 110°C with 6 M HCl in evacuated sealed tubes. Hydrolyzed samples were analysed on a Beckman model 120 C amino acid analyzer. Half-cystine was determined as cysteic acid after performic acid oxidation according to the method of Moore [10]. Tryptophan was determined by spectral analysis as described by Bencze and Schmid [11].

N-terminal analysis. N-terminal amino acid analysis was performed by the dansyl chloride procedure according to the method of Gray [12]. The dansyl derivatives were identified by thin-layer chromatography on polyamide sheets (Schleicher and Schüll, Dassel, G.F.R.) as described by Hartley [13].

Determination of thiol groups. Free SH groups were titrated with 5,5′-dithiobis (2-nitrobenzoic acid) by the method of Ellman [14].

Determination of sugar content. Neutral sugars were estimated by the anthrone method of Dreywood [15] as modified by Roe [16], using galactose as standard. For characterisation of neutral sugars, the enzymes were hydrolyzed at 100°C with 1 M HCl for 3.5 h in sealed, N_2-flushed tubes, followed by gas-liquid chromatography of the trimethylsilyl derivatives [17].

Hexosamines were determined by the Elson-Morgan reaction [18] after hydrolysis of the protein in 4 M HCl at 100°C for 4 h in sealed tubes under vacuum. Sialic acid was assayed by the thiobarbituric acid procedure of Warren [19] after hydrolysis of the protein in 50 mM H_2SO_4 at 80°C for 1 h. *N*-acetylneuraminic acid was used as standard.

Results

Enzyme stability

Storage. The enzyme preparations could be stored for several months at 4°C and pH 5.4 in 50 mM sodium acetate buffer, without any loss of activity. In addition, there was not detectable loss in enzymic activity when either enzyme was kept in diluted solutions in the same buffer (<10 μg/ml). Repeated cycles of freezing and thawing did not affect the activity of fucosidase II, but 30% of the activity of fucosidase I was lost after 3 cycles.

Thermal stability. The thermal stability of the two enzymes was tested at pH 5.4 in 100 mM acetate buffer. As shown in Table I, fucosidase II was heat-

TABLE I

THERMAL STABILITY OF β-FUCOSIDASES I AND II

The experiments were performed with enzyme solutions (100 μg/ml) in 100 mM acetate buffer at pH 5.4 maintained for 5 min at the desired temperature. After incubation, aliquots were withdrawn and the residual activities were quickly determined with *p*-nitrophenyl-β-D-galactoside (PNPGal), *p*-nitrophenyl-β-D-glucoside (PNPGlc) and *p*-nitrophenyl-β-D-fucoside (PNPF) as substrates. The values reported are expressed as percentages of the initial activity determined in the standard conditions and are averages of 3 determinations.

Temperature (°C)	β-Fucosidase I			β-Fucosidase II		
	PNPGal	PNPGlc	PNPF	PNPGal	PNPGlc	PNPF
37	100	100	100	100	100	100
47	91	92	89	100	100	99
53	40	38	40	73	73	72
55	17	17	18	50	50	50
57	3	4	4	16	16	15
60	0	0	0	0	0	0

stable over a wider temperature range than fucosidase I. The activity decay was found to be the same for all tested synthetic substrates. These results are in good agreement with the presence of a single molecule species with β-galactosidase, β-glucosidase and β-fucosidase activities as previously shown for each one of the two enzymes by mutual competition studies between substrates and inhibition experiments with aldonolactones and glycopyranoses [2].

pH stability. The enzyme stability at various pH values was followed using various 0.05 M citric acid/0.1 M sodium phosphate mixtures. After incubation at 37°C for 1 h, the preparations were adjusted to pH 5.4 and the remaining activity was measured by the standard method. Under these conditions, fucosidase I was found to be stable in the pH range 5.5—7.0 and fucosidase II between pH 5.0 and 7.5.

Denaturating agents. When the enzymes were incubated at 25°C in 6 M urea, the activity of fucosidase I was much more rapidly lost than that of fucosidase II. When urea was gradually removed by dialysis against a 100 mM sodium acetate buffer (pH 5.4) at 4°C for 24 h with 3 buffer changes, more than 50% of the original activity of fucosidase II was recovered. This apparent renaturation dropped to 25% with fucosidase I. Both enzymes were completely and irreversibly inactivated in the presence of 0.1% (w/v) SDS.

Activation energy

As shown in Fig. 1, the logarithms of the initial reaction rate varied linearly with $1/T$ between 28°C and 49°C. The calculated values of the activation energy were very similar: 12.3 and 12.4 kcal/mol for fucosidases I and II, respectively.

Molecular weights of the enzymes and enzyme subunits

Gel filtration. The apparent molecular weights of fucosidases I and II were about 360 000 and 120 000, respectively. The Stokes' radii of the molecules calculated by the method of Ackers [20] are 75 and 44 Å.

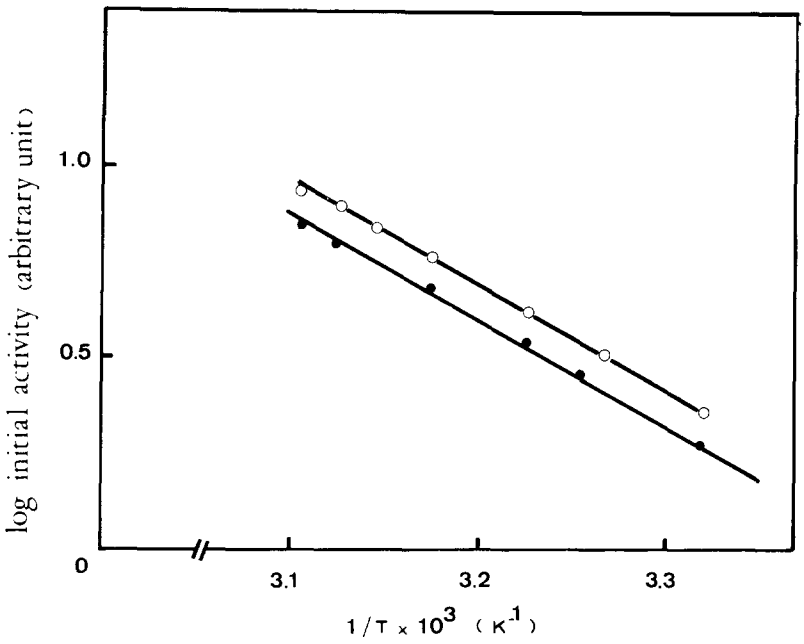

Fig. 1. Arrhenius plots for β-fucosidase I (●———●) and β-fucosidase II (○———○). Logarithms of reaction velocities were plotted against reciprocals of temperatures.

Ultracentrifugation. Ultracentrifugation analysis showed, for each enzyme, a single component with sedimentation coefficients of 10.2 S and 6.0 S for fucosidases I and II, respectively. The above value of the Stokes' radius (a) and that of the sedimentation coefficient (s) were employed to estimate the molecular weight using the modified Svedberg equation:

$$M = \frac{6\pi\eta Nas}{1 - \overline{v}\rho}$$

where η is the viscosity of the medium, N Avogadro's number, ρ the density of the solvent and $\overline{v}$ the partial specific volume of the protein (determined by summation of the individual values found in the literature for the amino acids and sugars [21]). The calculated values of $\overline{v}$ for fucosidases I and II were 0.719 ml/g and 0.727 ml/g, respectively. From these data, the molecular weights were estimated to be in the range 300 000—330 000 (fucosidase I) and 105 000—120 000 (fucosidase II), assuming an error of 2% on calculated $\overline{v}$ values.

SDS gel electrophoresis. It has previously been shown that native forms I and II migrated as a single species in gel electrophoresis at pH 8.5 [2]. To obtain information about the possible presence of subunits, electrophoresis assays on SDS polyacrylamide gels were performed. Fucosidase II again showed a single protein band corresponding to an apparent molecular weight of 110 000 ± 5000. This value is in good agreement with that derived from gel filtration and it suggests a monomeric structure for this enzyme. Under exactly the same conditions, fucosidase I yielded two bands consistent with the presence of 2 subunits of about 170 000 and 190 000 daltons.

Amino acid composition

The amino acid composition of the two enzymes is reported in Table II. The results are the mean of three complete analyses performed on three preparations obtained independently. They are recalculated on the basis of mol. wt. = 100 000 in order to facilitate the comparison with other known β-glycosidases. The contribution of some groups of residues to the total amino acid composition was compared with the average values calculated by Dayhoff [22] from 108 known proteins. As shown in Table III, both enzymes are especially rich in acidic and aromatic residues. Other enzymes of the same group, such as the β-galactosidase from *Escherichia coli* [23] and the β-glucosidase from the cytosol of rat kidney cortex [24] are known to yield similar results in this respect.

Moreover the ratio of hydrophilic over apolar amino acids estimated according to Hatch [25] was found to be lower for fucosidases I and II (1.29) than the average for a number of other proteins (1.45). This result suggests a comparatively compact structure, stabilized by hydrophobic bonds.

Sulfhydryl groups

The amino acid analyses presented above show the presence of 8.2 ± 0.2 and 4.0 ± 0.2 residues of half-cystine per 100 000 g protein for fucosidases I and II, respectively. Since approximately the same number of free SH groups were found with Ellman's reagent, it is tempting to speculate that the enzyme do not contain S-S bridges. Further investigations are in progress to confirm this assumption.

TABLE II

AMINO ACID COMPOSITION (NUMBER OF RESIDUES PER 10^5 g/PROTEIN)

Amino acid	β-fucosidase		β-galactosidase from *E. coli* *	β-glucosidase from rat kidney **
	I	II		
Asx	104.7	120.9	91.3	89.1
Thr	40.9	42.4	48.2	38.1
Ser	53.7	48.6	49.7	39.7
Glx	72.8	60.6	105.5	109.5
Pro	36.2	33.5	49.5	64.1
Gly	70.4	65.9	63.1	60.9
Ala	55.9	66.3	69.0	56.9
Cys	8.2	4.0	14.1	17.9
Val	50.6	61.4	55.6	40.9
Met	7.8	6.1	17.8	12.4
Ile	57.6	49.4	35.6	42.2
Leu	72.4	77.9	81.6	61.0
Tyr	54.2	49.8	26.6	33.3
Phe	46.1	42.6	33.4	57.4
His	24.0	19.2	26.7	17.4
Lys	44.1	52.0	21.5	44.0
Arg	35.5	36.2	54.8	42.2
Trp ***	34.0	36.9	26.0	26.0

* Data from Craven et al. [23].

** Data from Glew et al. [24].

*** Tryptophan was determined spectrophotometrically [11].

TABLE III

CONTRIBUTION OF SOME GROUPS OF AMINO ACIDS TO THE TOTAL COMPOSITION (AVERAGE WEIGHT %)

Group	β-fucosidases		β-galactosidae from *E. coli* *	β-glucosidase from rat kidney **	Average values of proteins ***
	I	II			
Acidic: Asx + Glx	21.5	21.7	24.1	24.4	9.6
Basic: Lys + Arg + His	14.4	14.9	15.0	14.6	13.2
Aromatic: Tyr + Phe + Trp	21.9	21.2	14.1	18.7	8.2
Apolar: Leu + Ile + Val + Phe + Met	27.6	27.6	26.0	25.8	23.9

* Calculated from data of Craven et al. [23].
** Calculated from data of Glew et al. [24].
*** Average values determined by Dayhoff [22] from known proteins.

N-terminal amino acid

After dansylation, the two enzymes were each found to possess a N-terminal alanine residue.

Sugars

The presence of carbohydrates in fucosidases I and II was detected by the periodic acid-Schiff staining after polyacrylamide gel electrophoresis [2]. Both enzymes contained the neutral sugars, galactose, glucose, mannose and fucose, the content being higher for fucosidase I (15.5% w/w) than for fucosidase II (7.5% w/w).

Amino sugars were also determined. The maximum release of hexosamines was obtained by hydrolysis in 4 M HCl at 100°C for 4 h in sealed tubes under vacuum. The amino sugar content determined by the method of Elson-Morgan was 6.1% (w/w) and 5.2% (w/w) for fucosidases I and II, respectively. No sialic acid was found in either enzyme.

Discussion

This work reports on some physicochemical and structural properties of two β-glycosidases purified to homogeneity from the digestive juice of an African giant snail, *Achatina balteata*. Due to their high affinity and activity on β-D-fucosides, these enzymes could be called β-D-fucosidases, although their activity on β-D-galactosides and β-D-glucosides is quite significant [3].

The two preparations yield a single band by electrophoresis in a non-dissociating medium and also a single N-terminal residue by dansylation. This state of purity, which is certainly higher than that so far attained for other members of the group, permits a first appreciation of the chemical structure of a β-D-fucosidase and also a comparison at the structural level with other well characterized β-glycosidases such as the β-galactosidase from *E. coli* [23] and the β-glucosidase from rat kidney [24]. All have in common a relatively high proportion of acidic and aromatic residues. But, the snail β-fucosidases seem to be devoid of disulfide bridges, the number of half-cystine residues found after

acid hydrolysis being equal, within experimental errors, to that of the free SH groups accessible to Ellman's reagent. This absence does not impair the thermal stability of the molecules, due to the probable existence of a number of intramolecular hydrophobic interactions.

The two snail β-fucosidases are glycoproteins as shown by periodic acid-Schiff staining after gel electrophoresis and by the quantitative determination of the individual sugars. The total sugar content was higher for β-fucosidase I (21.6% w/w) than for β-fucosidase II (12.7% w/w). No sialic acid could be detected in either enzyme. Similar results were reported by Got and Marnay [26] for two β-hexosidases isolated from *Helix pomatia*. The sugar content was 15—16% in this case. The necessity of a sugar moiety for exocellular enzymes has been postulated by Eylar [27].

In spite of this relatively high sugar content, concordant results were obtained for the molecular weights of the enzymes determined by gel filtration and ultracentrifugation. Glycoproteins possessing more than 10% of sugars have been occasionally observed to have abnormal behaviour during gel filtration [5, 21] and electrophoresis [28]. In contrast, no difficulties in this respect have been encountered in the case of enteropeptidase with about 40% sugars [29]. The length of the sugar chains and their positions at the molecule surface may be expected to play a role in the physicochemical properties of glycoproteins.

On the basis of this work, it is probable that the two forms of β-fucosidases are not structurally related proteins. Additional sequence and immunological studies are in progress to evaluate the degree of homology between the two enzyme molecules.

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