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KINETIC STUDIES ON β-FUCOSIDASES OF *ACHATINA BALTEATA*

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Summary

β-Fucosidases (β-D-fucoside fucohydrolase, EC 3.2.1.38) isolated from the digestive juice of *Achatina balteata* catalyze hydrolysis of β-D-fucosides, β-D-glucosides and β-D-galactosides but the values of kinetic parameters show that catalytic efficiency is maximum towards β-D-fucosides. The results of mixed substrate incubation studies and inhibition by glycopyranoses indicate that there is at least one site at which all tested substrates are hydrolyzed. In the absence of inhibitor, the reciprocal plots exhibit a significant downward curvature. If a substrate analogue is present, the plots can be straight lines. These results are consistent with the presence on the enzyme molecule of at least two distinct sites for the substrate molecules, one being an active site and the other being either a second active site with different kinetic parameters or a modifier site. Also data are shown to fit quite well with the mechanism proposed for a mnemonic enzyme.

Introduction

A few β-glycosidases have been shown to possess a wide substrate specificity with respect to β-galactosides, β-glucosides and β-fucosides [1—5]. These enzymes were considered as β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) or β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) in consideration of their respective affinity and activity towards various

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Abbreviations: pNPGal, *p*-nitrophenyl-β-D-galactosidase; oNPGal, *o*-nitrophenyl-β-D-galactoside; pNPGlc, *p*-nitrophenyl-β-D-glucoside; pNPFuc, *p*-nitrophenyl-β-D-fucoside; BrNapGal, 6-bromo-2-naphthyl-β-D-galactoside; BrNapGlc, 6-bromo-2-naphthyl-β-D-glucoside; BrNapFuc, 6-bromo-2-naphthyl-β-D-fucoside.

substrates. Two β-glycosidases, forms I and II, were recently isolated from the digestive juice of a giant African snail *Achatina balteata* [6]. On the basis of their molecular and physicochemical properties, it is probable that the two forms are not structurally related proteins [7].

Due to their high activity on β-D-fucosides, these enzymes which are glycoproteins can be called β-D-fucosidases (EC 3.2.1.38) although their activity on β-D-galactosides and β-D-glucosides is quite significant. The presence of at least one common site for the hydrolysis of all tested substrates was previously suggested for each of the two enzymes but the results did not allow us to determine whether each enzyme possesses one or several catalytic sites. Moreover, the preliminary assays have shown that these two β-fucosidases displayed abnormal kinetic behaviour. In this paper, a study of the kinetic properties is reported in order to obtain more information about these questions.

Materials and Methods

Enzymes. Purification of the two β-fucosidases isolated from the digestive juice of *A. balteata* was carried out as described previously [6]. Both enzymes were found to be homogeneous by polyacrylamide gel electrophoresis [6] and analytical ultracentrifugation [7]. Furthermore, when the preparations of each enzyme were rechromatographed on a Sephadex G-200 column, protein and activity peaks coincided exactly and were symmetrical. The specific activities of the fractions applied to the column were completely recoverable.

Reagents. Synthetic substrates: *o*-nitrophenyl-β-D-galactoside (ONPGal), *p*-nitrophenyl-β-D-galactoside (pNPGal), *p*-nitrophenyl-β-D-glucoside (pNPGlc) and *p*-nitrophenyl-β-D-fucoside (pNPFuc) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Glucostat was obtained from Worthington Biochemical Co. (Freehold, NJ, U.S.A.). All carbohydrates and other chemicals used were of analytical grade.

Enyme assays. Unless otherwise stated, determinations of nitrophenylglycosidase activities were carried out at 37°C under the following conditions of incubation: 100 mM sodium acetate buffer (pH 5.4) containing substrate at different concentrations and 50 μl of a suitable dilution of enzyme solution in a final volume of 2 ml. All components were prewarmed for 5 min at 37°C in a water bath. The reaction was stopped by adding 2 ml of 100 mM carbonate/bicarbonate buffer (pH 10.8). The extinction at 420 nm was measured and the quantity of liberated aglycone was determined from the appropriate calibration curve. For lactase activity, determined with lactose as substrate, the released glucose was determined with glucose oxidase (Glucostat). Whenever necessary, the observed rates were corrected for non-enzymatic hydrolysis of the substrate under the same experimental conditions. The apparent kinetic parameters (K_m and V) were determined from Lineweaver-Burk and Eadie plots. Each experimental point was determined at least in triplicate and in all cases the initial velocity was used for plotting. Agreement between the determinations is indicated by a single point on the figures.

One unit of enzyme is defined as that amount of enzyme which liberates 1 μmol of substrate per min under the experimental conditions described above. Specific activity is expressed as units per mg of protein. Protein was

determined by the method of Lowry et al. [8] using crystalline bovine serum albumin as standard or by measuring the absorbance at 280 nm. The value of the extinction coefficient $E^{1\,cm}_{1\,mg/ml}$ was 1.6 for β-fucosidase I and 2.2 for β-fucosidase II.

Results

Reactions catalyzed by the two enzymes

A variety of glycosides were tested for their ability to serve as substrates. Both enzymes are highly specific for the β-anomeric configuration of the glycosidic linkage. They catalyze essentially the hydrolysis of β-D-galactosides (oNPGal, pNPGal, BrNapGal, lactose), β-D-glucosides (oNPGlc, pNPGlc, cellobiose, BrNapGlc) and β-D-fucosides (BrNapFuc, pNPFuc). They can also hydrolyze β-D-xylosides but at a much lower rate. On the other hand, the two enzymes are completely free of β-glucuronidase, *N*-acetyl-β-D-glycosaminidase and arylsulfatase activities which were found to be very active in the crude digestive juice of *A. balteata* [9]. In the absence of identified physiological substrates, the best substrates are the synthetic ones especially if the ratios V/K_m are considered [10].

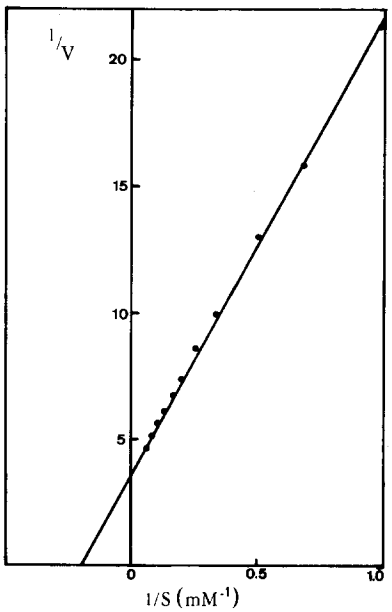
pH and temperature dependence

The effect of pH on the catalytic activity of the two enzymes was studied using *p*-nitrophenyl-β-D-glycosides as substrates. Enzymes were incubated with substrates in 100 mM sodium phosphate/50 mM citric acid buffers, pH 2.9—7.5 at 37°C. The pH curves had very similar shapes with activity maximum between pH 5.2 and 5.6. These values agree with the results obtained from crude digestive juice [9]. No difference in pH optimum was noticed using acetate buffer instead of phosphate/citrate buffer but in this case, the activity was slightly reduced indicating a small but significant effect of ions.

The activity of the two enzymes was measured at various temperatures between 25 and 60°C. The incubations were performed for 5 min according to the standard assay procedure. Under these conditions, the optimal temperature was the same for all the substrates and found to be 51—52°C for β-fucosidase I and 54—55°C for β-fucosidase II. The temperature coefficient Q_{10} determined between 30 and 40°C from the Arrhenius plots [7] was around 1.9 for both enzymes towards *p*-nitrophenyl-β-D-glycosides.

Effect of substrate concentration on enzyme catalysis

This investigation was carried out with five substrates, namely oNPGal, pNPGal, pNPGlc, pNPFuc and lactose. The concentration of substrates ranged from 0.1 to 19 mM for nitrophenylglycosides except for pNPFuc (0.1 to 16 mM) and from 1.0 to 180 mM for lactose. The experiments were performed at 37°C and pH 5.4 according to the standard assay procedure. Data were analyzed by the double-reciprocal method of Lineweaver-Burk and also by the Eadie plot. Only the kinetics of hydrolysis of pNPGal by β-fucosidase I (Fig. 1) and lactose by β-fucosidase II (Fig. 2) displayed a Michaelian behaviour. Lineweaver-Burk as well as Eadie plots revealed in the other cases two linear segments, the rupture in slope being observed at a substrate concentration of 2—8



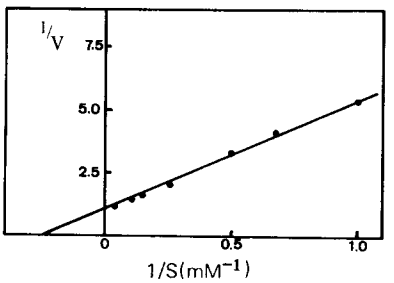


Fig. 1. Lineweaver-Burk plot of $1/v$ versus $1/S$ for the hydrolysis of pNPGal by β-fucosidase I. Initial velocity is expressed as μmol of *p*-nitrophenol liberated in the assay.

Fig. 2. Lineweaver-Burk plot of $1/v$ versus $1/S$ for the hydrolysis of lactose by β-fucosidase II. Initial velocity is expressed as μmol of glucose liberated in the assay.

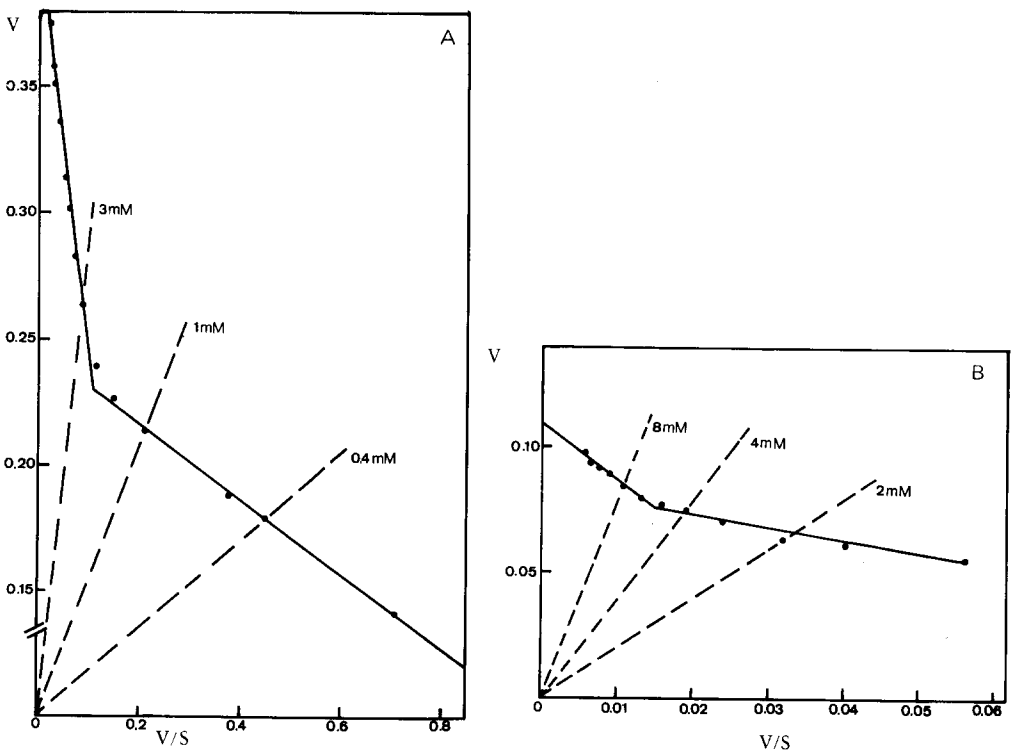


Fig. 3. Eadie plots for the hydrolysis of pNPGlc by β-fucosidase I (A) and by β-fucosidase II (B).

mM according to the substrate. An activation at high substrate concentration was observed. Due to their obvious deviation from the hyperbolic shape, the biphasic Eadie plots obtained for β-fucosidases I and II with pNPGlc as substrate are shown for illustration in Fig. 3A and B respectively.

Determination of kinetic parameters

The apparent kinetic parameters for the reactions catalyzed by the two enzymes have been determined at optimal pH value. For purposes of comparison, the apparent Michaelis constants were determined, in particular, for three substrates carrying the same aglycone in order to minimize any modifications in its effect on the lability of the linkage. Data are given in Tables I and II in which K_m^H and K_m^L are the Michaelis constants and V^H and V^L are the maximal velocities derived by extrapolation from the linear segments of the plots obtained for the high and low substrate concentrations.

Although the two enzymes appeared to be relatively unspecific, the examination of these tables indicates that their enzyme specificity is largely dependent on the nature of the substrates assayed. For example, in the case of β-fucosidase II at low [S] which determine the primary specificity, the V^L/K_m^L ratio, known to be the more significant parameter with respect to the catalytic efficiency, decreases by a factor of more than 900 from pNPFuc to lactose.

Whilst the values of V towards the synthetic substrates were comparable in magnitude, the values of V/K_m show clearly the importance of the glycosidic

TABLE I

APPARENT KINETIC PARAMETERS FOR THE HYDROLYSIS OF SOME GLYCOSIDES BY β-FUCOSIDASE I

The Michaelis constants and the maximal velocities determined from the linear segments of the plots for the low and high substrate concentrations are expressed respectively as mM and units/mg protein.

Substrate	K_m^L	K_m^H	V^L	V^H	V^L/K_m^L	V^H/K_m^H
o-Nitrophenyl-β-D-galactoside	3.3	8.7	31	56	9.4	6.5
p-Nitrophenyl-β-D-galactoside	5.0	5.0	25	25	5.0	5.0
p-Nitrophenyl-β-D-glucoside	0.2	1.6	23	35	115	22
p-Nitrophenyl-β-D-fucoside	0.1	0.4	50	60	500	150
Lactose	2.8	7.2	9	18	3.2	2.5

TABLE II

APPARENT KINETIC PARAMETERS FOR THE HYDROLYSIS OF SOME GLYCOSIDES BY β-FUCOSIDASE II

The Michaelis constants and the maximal velocities determined from the linear segments of the plots for the low and high substrate concentrations are expressed respectively as mM and units/mg protein.

Substrate	K_m^L	K_m^H	V^L	V^H	V^L/K_m^L	V^H/K_m^H
o-Nitrophenyl-β-D-galactoside	2.5	6.1	71	93	28	15
p-Nitrophenyl-β-D-galactoside	1.5	2.5	46	59	30	24
p-Nitrophenyl-β-D-glucoside	0.6	2.2	40	52	67	24
p-Nitrophenyl-β-D-fucoside	0.04	1.3	54	63	1350	48
Lactose	4.0	4.0	5.8	5.8	1.4	1.4

moiety of the substrate in the enzymic specificity. The single replacement of the methyl by a hydroxymethyl group on the carbon 5 of the β-fucoside, reduces the value of this ratio by a factor of about 100 and 50 for β-fucosidases I and II, respectively.

Finally the values of kinetic parameters indicate a high binding specificity of the two enzymes towards the β-D-fucoside. For both enzymes, the relative affinity for glycosyl residues can be classified in the following decreasing order: β-D-fucosyl > β-D-glucosyl > β-D-galactosyl.

Evidence for the hydrolysis of β-galactosides, β-D-glucosides and β-D-fucosides at a common catalytic site

The presence on each of the two enzymes of a common site catalyzing the hydrolysis of β-D-galactosides, β-D-glucosides and β-D-fucosides has been previously suggested [6]. To confirm this assumption, mutual competition studies between substrates have been performed in two ways.

First, mixed-substrate incubation studies were performed with various concentrations of nitrophenyl-β-D-glycosides by using fixed concentrations of lactose as inhibitor. As shown by measuring the release of nitrophenol, the hydrolysis of nitrophenylglycosides was inhibited by lactose competitively. Moreover, the K_i values (4.2—4.4 mM) of lactose obtained for these different assays are very close to its Michaelis constant (4.0 mM) when it is used as substrate in the case of β-fucosidase II. For β-fucosidase I, the K_i values of lactose (6.6—7.4 mM) agree with its K_m value (7.2 mM) extrapolated from the linear segment of the plots obtained for high substrate concentrations.

Secondly, kinetic experiments involving two *p*-nitrophenyl-β-D-glycosides were also carried out. For these experiments, substrate concentrations were chosen so as to be within the linear portion of the curves corresponding to K_m^H and V^H. The results are treated by the Dixon method [11]. As shown in Table III, the values observed for total velocity of hydrolysis agreed quite well with the theoretical ones calculated from the common site reaction and disagreed

TABLE III

MIXED-SUBSTRATE ANALYSIS ON *p*-NITROPHENYL-β-D-GLYCOSIDES

Studies were made using three different combinations of substrates as follows: pNPGal + pNPGlc, pNPGal + pNPFuc and pNPGlc + pNPFuc. The values calculated from the reaction due to distinct sites were obtained by a simple summation of the hydrolytic rate of each substrate and the values calculated assuming a common site determined by the Dixon equation [11].

Enzyme	pNPGal (5 mM)	pNPGlc (5 mM)	pNPFuc (8 mM)	Rate of *p*-Nitrophenolformed (arbitrary units)		
				Calculated		Observed
				Distinct sites	Common site	
β-Fucosidase I	0.390	0.850	—	1.240	0.840	0.900
	0.132	—	0.730	0.862	0.710	0.720
	—	0.265	0.571	0.836	0.542	0.535
β-Fucosidase II	0.375	0.380	—	0.755	0.450	0.430
	0.450	—	0.640	1.090	0.647	0.620
	—	0.542	0.813	1.355	0.805	0.815

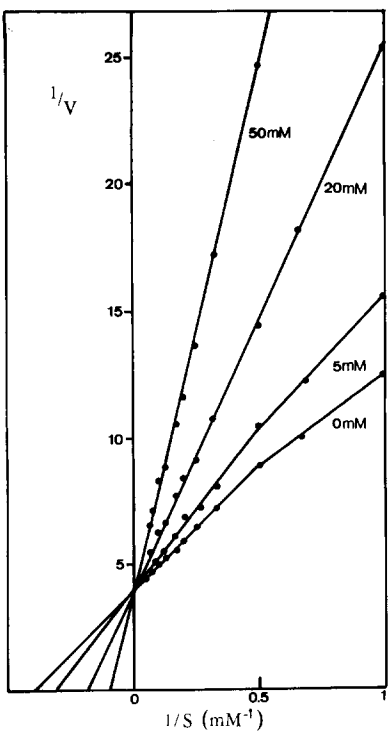
with those calculated from a reaction scheme involving distinct sites (one per substrate).

Modification of kinetic properties

Glycopyranoses (galactose, glucose and fucose) have been shown to inhibit reactions catalyzed by the two enzymes [6]. In the absence of these inhibitors, a substrate activation is observed. The downward curvature obtained from the double reciprocal plots can be decreased in the presence of glycopyranose as illustrated in Fig. 4. At high concentration of glycopyranose, Michaelis-Menten kinetics are observed over the entire substrate concentration range. Consequently, the substrate activation curve in the presence of the substrate alone extrapolates to the value of V determined at high inhibitor concentration (Fig. 5).

Differential effects of ions and other effectors on the two enzymes

A variety of ions were tested for their effect on the activity of the two enzymes. As shown in Table IV, β-fucosidase I was slightly more affected than β-fucosidase II by the presence of ions. No cation was found to be activating.



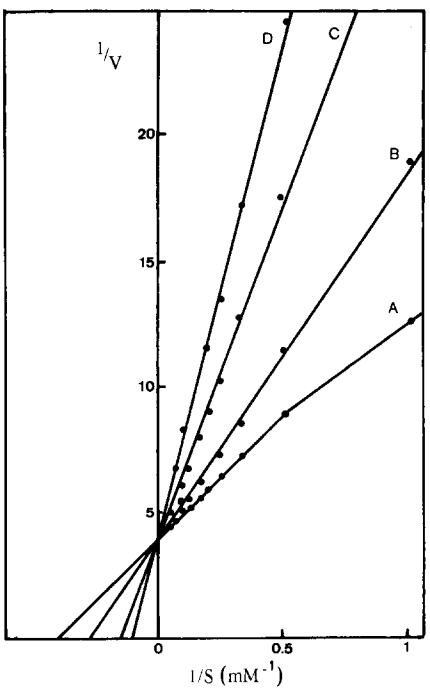


Fig. 4. Double reciprocal plots showing the effect of fucose at various concentrations on the hydrolysis of pNPGal by β-fucosidase II.

Fig. 5. Double reciprocal plots for the hydrolysis of pNPGal by β-fucosidase II in the absence of glycopyranose (curve A) and in the presence of 100 mM galactose (curve B), 100 mM glucose (curve C) and 50 mM fucose (curve D).

TABLE IV

EFFECT OF VARIOUS IONS ON THE ACTIVITY OF TWO β-FUCOSIDASES

The values reported are expressed as percentage of the initial activity determined in the standard conditions and are averages of four determinations.

Substance added (50 mM)	Enzyme activity (% of control)		Substance added (50 mM)	Enzyme activity (% of control)	
	β-Fucosidase I	β-Fucosidase II		β-Fucosidase I	β-Fucosidase II
None	100	100	none	100	100
K^+	100	100	Cl^-	100	100
Ba^{2+}	97	100	CH_3COO^-	100	100
Mg^{2+}	95	100	SO_4^{2-}	103	103
Ca^{2+}	88	98	SO_3^{2-}	98	108
Mn^{2+}	80	94	Citrate	102	103
Co^{2+}	50	78	$H_2PO_4^-$	101	103
Ni^{2+}	53	75	IO_3^-	98	104
Zn^{2+}	13	28	I^-	93	98
Cu^{2+}	0	0	F^-	80	90

Moreover the activity of the two enzymes was neither inhibited nor stimulated by EDTA at a final concentration of 50 mM. The lack of inhibition by EDTA as well as the lack of stimulation by cations strongly suggests that no metal ion is intimately involved in the catalytic process. These data are very different from those reported for a number of β-glycosidases for which metal ions play an essential role in the enzymic hydrolysis [3,12—14].

Tris is known to be competitive inhibitor for mammalian glycosidases [15]. Similarly with β-fucosidase II, the inhibition by Tris is competitive with respect to all the substrates. In contrast, with β-fucosidase I, the inhibition is of the non-competitive type.

Discussion

β-Fucosidase activity has been detected in a wide variety of biological materials [16—18] including the snail digestive juice [2,9]. It is generally assumed that this activity occurs in association with β-glucosidase [4] and/or β-galactosidase activities [19—21]. But, in the absence of pure enzyme, no definitive conclusion could be drawn as to the relationship between these activities. Recently, two β-glycosidases from *A. balteata* have been purified to homogeneity as demonstrated by the usual criteria of purity. Both enzymes are highly specific for the β-anomeric configuration of the glycosidic linkage. They catalyze the hydrolysis of various derivatives of β-D-galatose, β-D-glucose and β-D-fucose but not that of the corresponding α-D-glycosides. As demonstrated by mutual competition studies between substrates and inhibition experiments, all the hydrolyzed substrates are recognized at least by a common catalytic site. However, the kinetic parameters at pH optimum are markedly different for the tested substrates. Our results indicate a high binding specificity of the two

enzymes towards the β-D-fucoside. The relative affinity for glycosyl residues can be classified for both enzymes in the following decreasing order: β-D-fucosyl > β-D-glucosyl > β-D-galactosyl. Moreover, fucose is a more effective inhibitor than galactose or glucose not only for β-fucosidase activity but also for β-glucosidase and β-galactosidase activities. The structural similarity between D-galactose and D-fucose (6-deoxygalactose) has suggested a genetic and structural relationship between β-D-galactosidase and β-D-fucosidase [16,22] but at least in the enzymes investigated so far, the β-D-fucosidase activity was not the main one. To our knowledge, this is the first time that highly purified enzymes from animal origin exhibited such a β-D-fucosidase activity. From the data reported in the present paper, these enzymes make good representatives of the Enzyme Nomenclature entry EC 3.2.1.38, deleted in the Recommendations of the Commission on the Enzyme Nomenclature in 1972 [23] and reestablished in 1978 [24].

Difficulties in the interpretation of kinetics have been shown to arise in a number of β-glycosidases due to atypical plots of the data. In a few cases, non-Michaelian kinetics could be explained by the presence in the preparations of at least two enzyme species with the same catalytic function but different kinetic parameters [25,26]. This assumption to interpret the anomalous kinetics observed in this work can be reasonably ruled out on the basis that the two enzymes were found to be homogeneous and also on the grounds that similar kinetic experiments repeated with enzyme preparations obtained at different steps of purification always showed non-hyperbolic substrate saturation curves with an increase in the biphasic character of the Lineweaver-Burk plots for the purest preparations. The hyperbolic curves obtained in the presence of glycopyranose like fucose also rule out the possibility that the non-hyperbolic curves obtained in the absence of inhibitor are due to a mixture of two enzymes with different affinities for the substrate.

For other β-glycosidases, the presence of several binding sites for the substrate has been suggested. The hydrolysis of GM_1-ganglioside by one of two β-galactosidase isoenzymes from human liver exhibited a non-hyperbolic substrate saturation curve. To explain the upward-curving Lineweaver-Burk plots, Ho et al. [27] assumed that the substrate acts as a modifier for its own hydrolysis. Moreover the hypothesis was made that substrate activation is essential, i.e the catalytic site of the non-activated enzyme has no affinity for the substrate. As an explanation for the anomalous kinetics of human brain β-galactosidase and β-glucosidase, Lisman and Hooghwinkel [28] assumed that the enzyme molecule possesses two binding sites, a catalytic site and a modifier (activator) or second active site. Ramaswamy and Radhakrishnan [29] have also suggested that the lactase-phlorizin hydrolase complex from monkey small intestine might have two catalytic sites, a phlorizin site and a cerebroside site but both hydrolyzing lactose. No deviation from activity versus lactose concentration was observed by these authors but it is possible that the range of substrate concentrations tested was not wide enough. Another explanation could be that the kinetic parameters of two catalytic sites towards lactose are not significantly different. The possible existence of more than one catalytically-active binding site in the enzyme molecule has also been suggested for other glycosidases such as specific α-galactosidase from human liver [30],

glucoamylase from rat small intestine [31] and acid α-glucosidases from rat liver [32] and rabbit muscle [33].

Our results can be interpreted in the same manner, i.e. the possibility that there are at least two distinct sites for the substrate molecules, one being an active site and the other one being either an active site with different kinetic properties or a modifier site. The mechanism which describes such a situation has been developed extensively by Frieden [34]. However, non-linear reciprocal plots with apparent substrate activation must be interpreted with circumspection. In particular, the existence of possible impurities in the substrate may affect the enzyme reaction [35]. Here, this eventually is not very plausible because demonstration of a hyperbolic and non-hyperbolic substrate saturation kinetic with the same substrate (pNPGal) for β-fucosidases I and II, respectively, eliminates the role of such impurities as a contributing cause to abnormal kinetics. The possibility of measuring too large a percentage of the reaction, thereby not measuring initial velocities can also be rule out since the assay system is always closely controlled.

Examination of the β-fucosidase I and II atypical kinetic behaviour is of great interest because β-fucosidase II and not β-fucosidase I appears to be monomeric although the two enzymes display the same abnormal kinetics. In consideration of this fact, negative cooperativity [36] which involve double reciprocal plots with concave downward but also an enzyme molecule containing subunits cannot be retained to interpret properly our data. Nevertheless, it is well known that a monomeric enzyme can exhibit atypical kinetics and has a behaviour similar to that of an allosteric enzyme. The mnemonical enzyme concept has been proposed recently by Ricard et al. [37] to explain the departure from Michaelian behaviour of such enzymes. This attractive model relies on the existence of conformational transitions which occur far from pseudo-equilibrium conditions. This type of conformational transition, called in this model 'mnemonical transition' requires the existence of a sort of enzyme memory, that is when the last product is released from the active site. The enzyme relaxes relatively slowly from one conformation to another, it 'remembers' for a while the state stabilized by the product. This permits homotropic interactions between two binding processes that are separated by time rather than space. This model could explain our results. In the absence of inhibitors, the reciprocal plots exhibit a significant downward curvature. At high concentration of substrate analogues or products in the reaction medium, the enzyme takes the conformation stabilized by the product and the plots turn into straight lines. Wheat germ hexokinase [38], rat liver glucokinase [39] and probably octopine dehydrogenase of *Pecten maximus* L. [40] are the mnemonical enzymes so far described. Kinetic results showed in this paper fit quite well with the model expected for a mnemonical enzyme. Nevertheless, other experiments such as transient kinetic studies must be undertaken to obtain further evidence of such a model for β-fucosidases.

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