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EVIDENCE FOR A SINGLE CATALYTIC AND TWO BINDING SITES IN THE ALMOND EMULSIN β-D-GLUCOSIDASE MOLECULE

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SUMMARY

An isoenzyme of glucosidase- isolated from sweet almond emulsin - and composed of a β-D-glucosidase, a β-D-galactosidase and a β-D-fucosidase, has been shown to possess β-D--xylosidase activity, as well. On the basis of the following results it has been concluded that the β-D-glucosidase and β-D-galactosidase activities reside in one catalytic site, but there are two kinetically distinst binding sites in the active center: 1./D-Glucono-1,5-lactone is shown to excert competitive inhibition on the hydrolysis of β-D-glucopyranoside and non-competitive inhibition on the hydrolysis of β-D-galactopyranoside. 2./ D-galactono-1,5-lactone competitively inhibits the hydrolysis of β-D-galactopyranoside, but possesses non-competitive inhibition on the hydrolysis of β-D-glucopyranoside. 3./ When the enzyme is incubated with two p-nitrophenyl glycoside substrates at or above their respective K_m values, the rate of p-nitrophenol formation is not additive but rather it is equal to the value calculated from the individual K_m values and relative maximum rates.

INTRODUCTION

Glucosidases with different stereospecificity for substituents at the C-4 position of the pyranose ring have been investigated by various research groups (1,2). Almond emulsin glucosidase preparations have been shown to hydrolize both β-D-glucopyranosides and β-D-galactopyranosides (1,2,3). However, Escherichia coli β-D-galactopyranosidase (4,5) and yeast β-D-glucosidase / β-D-glucoside glucohydrolase, EC 3.2.1.21/ (6) are stereospecific for substituents at the C-4 position. It has been shown that the dual activity of almond emulsin enzyme is connested with the presence of the same protein moiety (1-3, 7-11).

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There are, however, opposite views if there is a single or two catalytic sites in the enzyme molecule responsible for both activities. On the basis of inhibition studies, Grover and Cushley (12) have proposed the existence of two catalytic sites, one for the β-glucosidase and an other for the β-galactosidase activity.

On the contrary, Heyworth and Walker (1), and recently Walker and Axelrod (13) have proposed a single catalytic site for both activities.

These contradictory results promted us to investigate the above enzyme reactions using the purified enzyme, prepared according to the procedure of Grover et al. (10). On the basis of these investigations a new proposal is advenced for the catalytic and binding sites of the two activities, dissolving the contradictions mentioned above. We have found that there are two binding sites, one for β-D-glucopyranoside - inhibited competitively by glucono-1,5--lactone and non-competitively by galactono-1,5-lactone - and one for β-D-galactopyranoside - inhibited competitively by galactono-1,5-lactone and non-competitively by glucono-1,5-lactone -, and also a single catalytic site confirmed by the results of mixed substrate examinations.

METHODS

Enzyme purification - Emulsin β-D-glucosidase / β-D-glucoside glucohydrolase, EC 3.2.1.21/ was prepared from Sweet almonds (18) and purified as described by Grover et al. (10). The enzyme was shown to be homogeneous by disc gel electrophoresis at pH 8,9.

Enzyme assays - β-D-glucosidase, β-D-galactosidase and β-D--xylosidase activities were measured in 50 mM sodium acetate buffer, pH 6,0 at 37°C. The total reaction volume was 5 ml. The reaction was initiated by addition of the substrate and the hydrolysis was proceeded for 20 min. The reaction was frozen by the addition of 1 ml of the reaction mixture to 2 ml of 0,5 M sodium hydroxide. The p-nitrophenolate ion concentration was measured spectrophotometrically at 400 nm, using a calibration curve. Substrate concentration ranges for p-nitrophenyl - β-D-glucopyranoside, p-nitrophenyl - β-D-galactopyranoside and p-nitrophenyl - β-D-

Table I. Km and V values of various β-glycosidases[a]

Substrate	Km/mM/	V/nmol/min/
p-Nitrophenyl-β-D-glucoside	8	40
p-Nitrophenyl-β-D-galactoside	80	40
p-Nitrophenyl-β-D-xyloside	9,3	1,0

[a] Values were determined from Lineweaver-Burk plots.

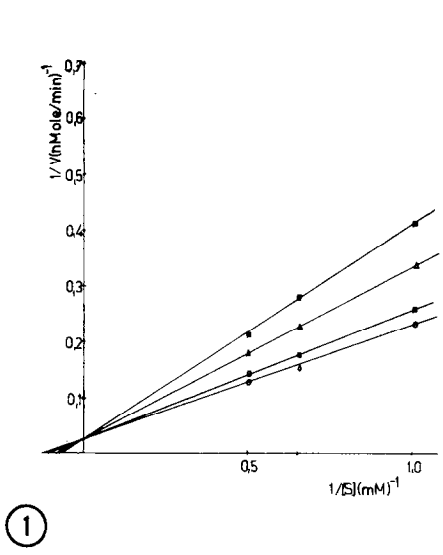
-xylopyranoside were o,25 to 2, 2,5 to 2o and 1,1 to 11 mM, respectively. The inhibitors, when used, were freshly dissolved in 5omM acetate buffer /pH=6/. Data were determined from Lineweaver Burk plots, fitted by a last-squares treatment.

Protein estimation - Protein content of the solutions was estimated by measuring the absorbance at 28o nm.

RESULTS

Xylosidase activity - The enzyme possesses in addition to the well known β-glucosidase, β-galactosidase and β-fucosidase activity also β-xylosidase activity. For comparison, the activity towards p-nitrophenyl-β-D-glucopyranoside and p-nitrophenyl-β-D-galactopyranoside was measured. The K_m and relative V values for the various substrates are given in Table I. Despite the relatively high affinity of the xyloside to the enzyme, the lack of the hydroxymethyl group at C-5 prevents the effective hydrolysis. The reason for this might be either the increased rotational freedom of the sugar moiety preventing its proper alignment to the catalytic site, or, the inability of the substrate for bringing the catalytic functions into the correct position by an induced fit mechanism.

Inhibition by glyconolactones - Investigating the influence of glyconolactones on the β-glucosidase and β-galactosidase activities of the enzyme it was found that D-glucono-1,5-lactone inhibited the β-glucosidase activity competitively /Fig.1./ and the β-galactosidase activity non-competitively /Fig.2./.



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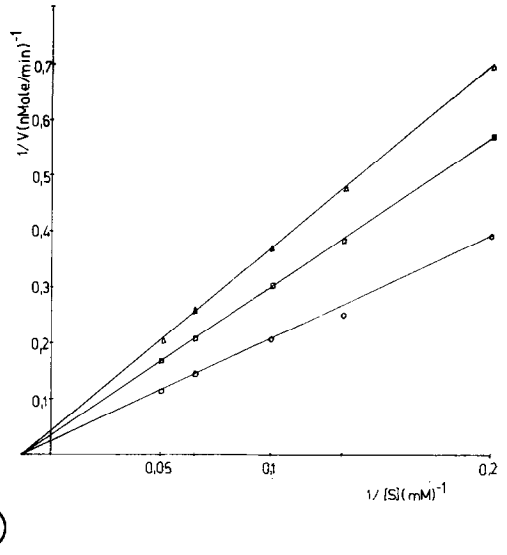

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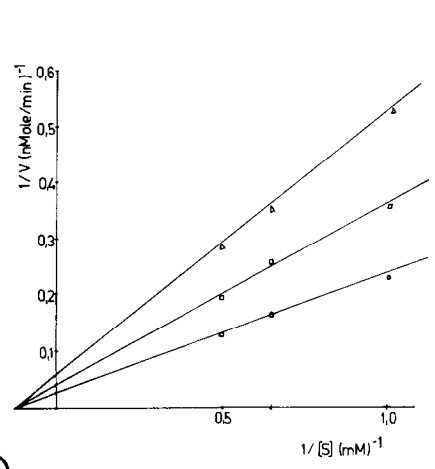
Fig. 1. Inhibition of β-D-glucosidase by D-glucono-1,5-lactone. Incubations and assays were as described under Materials and Methods. o——o I = 0 ; □——□ I = 0,084 mM ; Δ——Δ I = 0,168 mM ; ■——■ I = 0,336 mM.

Fig. 2. Inhibition of β-D-galactosidase by D-glucono-1,5-lactone. Incubations and assays were as described under Materials and Methods. o——o I = 0 ; □——□ I = 0,168 mM ; Δ——Δ I = 0,336 mM.

An opposite effect of D-galactono-1,5-lactone was found, inhibiting the β-D-glucosidase activity non-competitively /Fig. 3./ and the β-D-galactosidase activity competitively /Fig. 4./.

Mixed substrate incubations - Experiments involving two substrates simultaneously were carried out to investigate whether more than on catalytic site exist on an enzyme of multiple specificity (1, 14). If a single site exists the rate of the reaction in the presence of both substrates will be less than the sum of the respective activities determined separately. A substrate will act as a competitive inhibitor if a second substrate is introduced. In this case the K_i value for the first substrate



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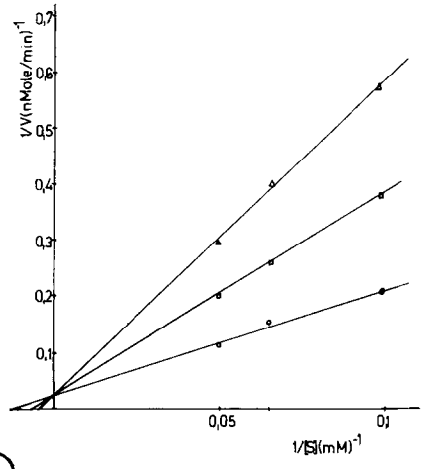

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Fig. 3. Inhibition of β-D-glucosidase by D-galactono-1,5-lactone. Incubations and assays were as described under Materials and Methods. o——o I = 0 ; □——□ I = 1,68 mM ; Δ——Δ I = 3,36mM.

Fig. 4. Inhibition of β-D-galactosidase by D-galactono-1,5-lactone. Incubations and assays were as described under Materials and Methods. o——o I = 0 ; □——□ I = 1,68 mM ; Δ——Δ I = 3,36 mM.

is equal to its K_m value and the rate of the hydrolysis can be given by the following equation /14/:

$$v = \frac{S_x V_x}{K_{mx}/1+S_y/K_{my}/+S_x} + \frac{S_y V_y}{K_{my}/1+S_x/K_{mx}/+S_y}$$

where S_x and S_y are the respective substrate concentrations, x and y, and K_m, V and v have their usual meanings. The knowledge of the values of K_m and V for both substrates permits a prediction of the rate when two substrates with known concentration are present.

The observed rates for the hydrolysis process are in good agreement with the calculated values, as shown in Table II.

Table II. Additive Substrate Effects on Almond Emulsin β-D-Glucosidase

Substrate	Rate of p-nitrophenol formed /nmol/min/	
	Observed	Calculated
5,0 mM p-nitrophenyl-glucoside /x/	23	
10,0 mM p-nitrophenyl-galactoside /y/	9,5	
x+y	18,3	17,2

Incubation and assays were as described under Materials and Methods. The incubation time was 5 min in each case.

DISCUSSION

It is generally accepted that almond emulsine β-glucosidase has both β-glucosidase and β-galactosidase activities. However, there are opposite theories whether there are independent catalytic sites responsible for the different activities. According to their mixed substrate incubation and inhibition studies Heyworth and Walker (1) found that both gluconolactone and galactonolactone competitively inhibit both activities. These authors, however, applied crude enzyme preparations for their investigation Lai and Axelrod (15) reported similar results for glucosylamine and galactosylamine, which - in the case of crude enzyme - are normally specific inhibitors of the corresponding glucosidases.

Recently two research groups have investigated the above problem in detail. Grover and Cushley (12) proposed the presence of two distinct catalytic sites on the basis of inhibition studies comparing the effect of inhibitors with the D-gluco- and galacto configuration, and using highly purified enzyme. The

gluco inhibitors were found to inhibit the β-glucosidase activity competitively but the β-galactosidase activity non-competitively. A reversed effect was shown for the galacto inhibitors.

On the contrary, on the basis of studies with highly purified enzyme, Walker and Axelrod (13) reported that the β-D-glucosidase, β-D-galactosidase and β-D-fucosidase activities were inhibited by each of the three corresponding glycosylamines in a competitive fashion. According to these results and also to those obtained in mixed substrate incubation investigations, they proposed a single catalytic site involved in the mechanism.

Our studies were carried out also with highly purified enzyme prepared by the method of Grover et al. /lo/. The results obtained with gluconolactone and galactonolactone, as binding site inhibitors, corresponds to those reported by Grover and Cushley /12/, namely, D-glucono-1,5-lactone inhibited the β-D--glucosidase activity competitively and the β-D-galactosidase activity non-competitively, whereas D-galactono-1,5-lactone inhibited the β-D-glucosidase activity non-competitively and the β-D-galactosidase activity competitively. The inhibitors were used in concentrations not as high as those applied by Grover and Cushley (12). According to these results two different binding sites seems to exist: one for the β-D-glucoside and an other for the β-D-galactoside.

Mixed substrate effects have been previously used by others to demonstrate the presence of a single catalytic site on glycosidases with multiple glycone specificity (1, 14). In our study the rate of the reaction, abtained with a combination of p-nitrophenyl-β-D-glucopyranoside and galactopyranoside, was in accordance with the rate equation for a single catalytic site. On the basis of our results we argue for the presence of two distinct

substrate binding sites and a single catalytic site on the β-D--glucosidase enzyme. The substrate binding sites are inhibited by binding site inhibitors, such as glyconolactones, whereas the catalytic site is inhibited by glycosylamines.

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