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STUDY ON THE ROLE OF TYROSINE SIDE-CHAINS AT THE ACTIVE CENTRE OF EMULSIN β-D-GLUCOSIDASE

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The role of exposed tyrosine side-chains in enzyme-catalyzed reaction by sweet almond emulsin β-D-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) has been studied using *N*-acetylimidazole as the specific reagent. The changes in activity, binding affinity and kinetic parameters (K_m, V) as a result of acetylation of the phenolic hydroxyl groups have been determined. The acetylation increased the K_m values of both β-glucosidase and β-galactosidase activities, whereas V remained unchanged. Similarly, the binding affinity for immobilized phenyl β-D-glucopyranoside decreased appreciably. After the removal of the acetyl groups the enzyme regained 96% of the original activity. It is concluded that the tyrosine moieties, located in the active centre of the enzyme, have both glucoside and galactoside binding functions.

Introduction

The function of the amino acid side-chains in the active centre of an enzyme can be studied by the application of specific reagents. In order to establish unambiguously whether the subsites present in the active centre play a role in the binding of the substrate and in the formation of products, or if they affect only the stability of the tertiary structure of the protein, the change in binding affinity (k_{-1}/k_{+1}) occurring upon modification must be known. The change in k_{-1}/k_{+1} due to modification can be determined by affinity chromatography using immobilized substrate, as a relatively simple spectrophotometric method. In a previous paper we have reported [1] that there are two substrate-binding sites and one catalytic site on the molecule of emulsin β-D-glucosidase (β-D-glucose glucohydrolase, EC 3.2.1.21).

Earlier studies have shown [2,3] that a carboxyl group is involved in the catalytic reaction, however, there is no information about amino acid moieties playing a role in the binding of the substrates. It is known that in the case of other glucosidases [4,5] tyrosine units are involved in the binding site.

The lack of detailed studies in the above field prompted us to investigate the role of tyrosine groups of the active centre of the enzyme in the enzyme reactions. *N*-Acetylimidazole has been reported [6] as agent for the acetylation of the hydroxyl groups of tyrosine. With this reagent the undesired side-reactions (mainly acetylation of the -SH and $-NH_2$ groups) can be avoided [7]. We now report on the application of this reagent for the emulsin β-D-glucosidase enzyme.

Methods

Enzyme purification. Emulsin β-D-glucosidase was prepared as described earlier [10].

Modification of the tyrosine moieties. Acetylation of the tyrosine hydroxyl groups of the enzyme was carried out at pH 6.5 in a 0.01 M phosphate buffer, since the enzyme and *N*-acetylimidazole are stable under these conditions. *N*-Acetylimidazole (analytical grade) was recrystallized from anhydrous benzene, dried in vacuo over phosphorous pentaoxide and kept

in a refrigerator. The reaction was performed with continuous stirring at 25°C using a pH-stat and enzyme concentration of 2 mg/ml. Solid *N*-acetylimidazole (300 molar excess) was added and aliquots (1 ml) were taken at intervals, the excess *N*-acetylimidazole was removed by chromatography on Sephadex G-25 columns (1.5 × 5 cm), equilibrated with pH 5.2 citrate-phosphate buffer and the same buffer was also used as the eluant. The protein was eluted in 3 ml fractions and the change of the β-D-glucosidase and β-D-galactosidase activities was determined.

Determination of the change in binding affinity. The change in binding affinity as a result of the chemical modification was determined by affinity chromatography using phenyl-β-D-glucopyranoside bound to Sepharose 4B [8]. The affinity gel (500 mg) was added to an enzyme solution (3 ml) of known absorbance (E_0) and the mixture was stirred at 4°C until equilibrium was attained (30 min). The mixture was allowed to stand until the sedimentation of the gel grains and the absorbance (E_m) was measured. The amount of enzyme bound to the gel was determined from the expression $100 \times E_0 - E_m/E_0 \times 500$ mg.

Determination of reaction kinetic constants. K_m and V values were determined using Lineweaver-Burk plots, as described [1].

Removal of the acetyl groups. On removal of the *O*-acetyl groups by treatment with 0.5 M hydroxylamine at pH 6.5 and 25°C for 30 min, 96% of the original activity of the enzyme (both β-glucosidase and β-galactosidase) was regenerated. The degree of acetylation was determined [9] according to the change in ultraviolet absorption at 278 nm during the deacetylation process. The number (n) of acetylated tyrosine units is given by the expression $\Delta E_{278} \times M_r/1160 \times c$, where ΔE_{278} denotes the difference of extinction measured at 278 nm, M_r is the molecular weight of the enzyme, c is the protein concentration of the enzyme (mg/ml) and 1160 is the molar extinction coefficient for *O*-acetyltyrosine.

Results

Changes in activity and binding affinity. The changes of the enzyme activity and the binding affinity on acetylation of the phenolic hydroxyl groups and the degree of acetylation are summarized in Table I. Under the chosen experimental conditions only the exposed tyrosine units are blocked [6].

TABLE I

CHANGES IN ACTIVITY, BINDING AFFINITY AND THE NUMBER OF MODIFIED TYROSINE UNITS DURING ACETYLATION

Incubation and assays were as described under Methods. n.d. not determined.

Time of modification (min)	Number of modified tyrosine groups	Remaining act.		Remaining binding affinity (%)
		β-glucosidase (%)	β-galactosidase (%)	
0	0	100	100	100
5	1.5	68.5	57	89
15	6	51.3	31	84
30	8	46.5	29	n.d.
40	9	44.5	21	64

For the emulsin β-D-glucosidase enzyme a molecular weight of 135 180 ± 770 was estimated [10] and nine tyrosine groups could be modified. Modification of six of the above nine tyrosine moieties caused a sharp decrease in both β-glucosidase and β-galactosidase activities as shown in Fig. 1. The modification

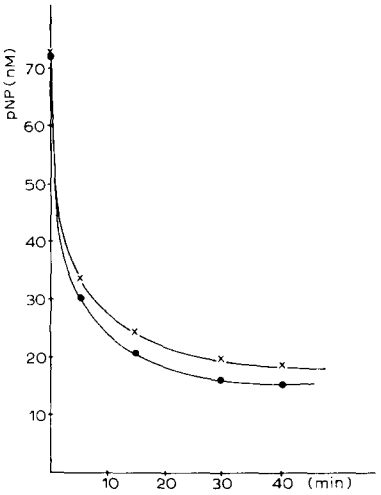

Fig. 1. Effect of acetylation of the exposed tyrosine side-chains of emulsin β-D-glucosidase. X———X, β-glucosidase activity; ●———●, β-galactosidase activity.

TABLE II

CHANGES IN KINETIC PARAMETERS DURING ACETYLATION OF THE EXPOSED TYROSINE GROUPS

Values were determined from Lineweaver-Burk plots.

Time of modification (min)	Number of modified tyrosine groups	K_m (Glc) (mM)	K_m (Gal) (mM)	V (Glc ; Gal) (nM/min)
0	0	8	80	40
5	1.5	12.3	114	40
15	6	20	154	40
30	8	32	266	40

of the remaining three tyrosine groups had only a slight influence on the activities.

The β-glucosidase activity and the binding affinity decreased to approx. half the original values, but the β-galactosidase activity showed larger decrease.

Changes in the kinetic parameters. The kinetic data for the modified enzyme (both β-glucosidase and β-galactosidase activities) were determined from Lineweaver-Burk plots fitted by least-squares treatment. The Michaelis constants (K_m) and V values are presented in Table II. The modification of tyrosine groups caused about a 3-fold increase of K_m, whereas V values remained unchanged.

Discussion

The foregoing data indicate that the tyrosine side-chains of the emulsin β-D-glucosidase enzyme have a substrate binding function. Acetylation of the exposed tyrosine groups led to the decrease of both catalytic activities. For the β-glucosidase activity a decrease of 55% was observed, whereas the β-galactosidase activity decreased to an even larger extent (79%). This latter observation is in good agreement with the fact that the β-D-galactoside binds much more weakly to the enzyme in the E-S complex than the β-D-glucoside (see K_m values in Table II).

The binding affinity, which is characteristic for the formation of an E-S complex decreased to a smaller extent (36%) in the case of β-glucoside. The changes in the kinetic parameters also prove that the phenolic hydroxyl groups play an important role in the binding of both substrates. On acetylation of the enzyme, the value of K_m increased 3-fold, whereas V values remained unchanged.

This suggestion is also supported by the results of deacetylation experiments. After removal of the acetyl groups 96% of the original activities was regenerated.

Evaluation of the obtained results by the method of Ray and Koshland [13] gave the relationships shown in Figs. 2 and 3. The plots of activity against time are not linear for both β-glucosidase (Fig. 2) and β-galactosidase (Fig. 3) activities. Each curve can be divided into two sections with different slopes, which can be extrapolated to zero. For the changes in activity the rate constants of inactivation characteristic of the modification of the different tyrosine residues can be determined from the slopes of the straight lines. These are for β-glucosidase activity $k_{i1} = 6.5 \cdot 10^{-2}$ min^{-1}; $k_{i2} = 6.25 \cdot 10^{-4}$ min^{-1} and β-galactosidase activity $k_{i1} = 0.1$ min^{-1}; $k_{i2} = 1.25 \cdot 10^{-3}$ min^{-1}.

From the values extrapolated to zero time it is concluded that, in the case of β-glucosidase activity the rate of inactivation caused by acetylation of 48% of the tyrosine groups (four) is much higher (10^2-fold) than that induced by acetylation of the other five groups. Similarly, in the case of β-galactosidase activity, acetylation of 30% tyrosine residues (three) causes much higher inactivation (10^2-fold).

Comparison of the results with those in Table I shows that three of the tyrosine groups are located in the active centre, and participate in the formation of the enzyme-substrate complex in both binding sites [1]. This assumption is supported by the observation that for the formation of the E-S complex, unsubstituted secondary hydroxyl groups have to be present on carbon atoms C-2, C-3 and C-4 [11]. Similar results were obtained recently by Roeser and Legler [14] in the case of β-glucosidase A_3 from *Aspergillus wentii.*

It is to be noted that, on acetylation, the β-glucosidase activity decreases to an extent of 55%. This is in agreement with earlier observations [11,12] that the aglycone part of the β-glucosides binds to a hydrophobic 'pocket' on the enzyme molecule. On the basis of these results it can be concluded that the aglycone and the glycosyl moieties bind to different binding sites and tyrosine side-chains take part only in the binding of the glycosyl moiety.

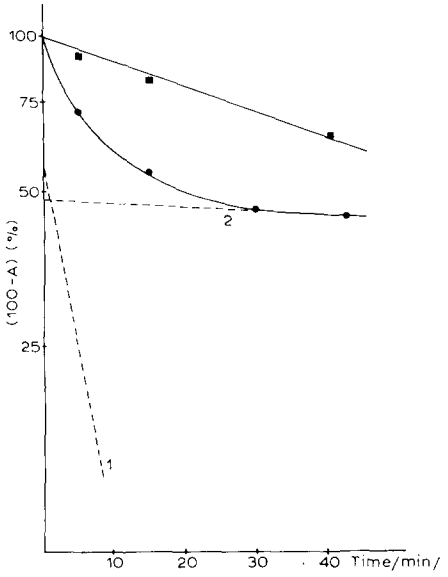

Fig. 2. Change in remaining β-glucosidase activity (●——●) and binding affinity (■——■) during acetylation of the exposed tyrosine side-chains of emulsin β-D-glucosidase.

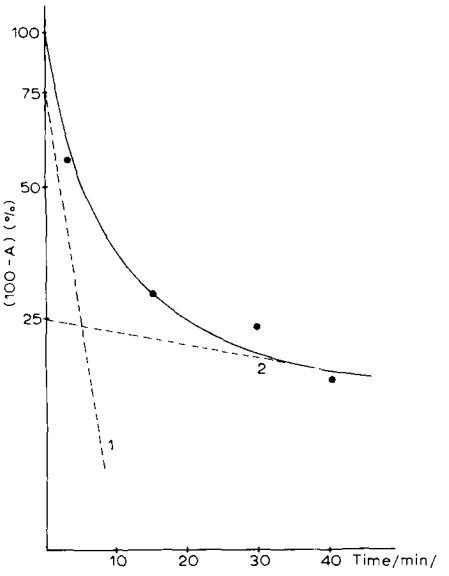

Fig. 3. Change in remaining β-galactosidase activity during acetylation of the exposed tyrosine side-chains of emulsin β-D-glucosidase.

The changes in binding affinity plotted vs. time afford a straight line, with a rate constant lower ($k = 9 \cdot 10^{-3}$ min^{-1}) than that characteristic of rapid inactivation. The relative low decrease (35%) caused by acetylation, may be explained by the fact that the hydrophobic interactions are stronger than the hydrogen bonding between the tyrosine groups and glycosyl moiety in the E-S complex. The tyrosine groups, however, play a very important role in the correct alignment of the glycosyl part with respect to the catalytic site, and thus the larger decrease of the activity (55%) is quite understandable.

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