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INACTIVATION OF β-FUCOSIDASE BY IODINE, *N*-ACETYLIMIDAZOLE AND TETRANITROMETHANE

EVIDENCE FOR THE EXISTENCE OF ESSENTIAL TYROSINE RESIDUES

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Summary

β-Fucosidase (β-D-fucoside fucohydrolase, EC 3.2.1.38) isolated from the digestive juice of *Achatina balteata* is markedly inactivated when the enzyme is preincubated with three different probes, specific for tyrosine residues in the experimental conditions used. An effective protection against inactivation of the enzyme is obtained in the presence of a substrate analogue. These data strongly suggest that β-fucosidase possesses essential tyrosine residues.

β-Glycosidases are very widely distributed in nature, being found in all organisms [1]. However, little is known about the mechanism of action and the active site of this enzyme group [2—3]. One of the reasons for this is the difficulty in obtaining sufficient quantities of homogeneous enzyme material. Moreover, in the animal kingdom, a number of glycosidases are frequently present in more than one molecular form giving rise to additional problems of separation. Recently, two β-glycosidases, forms I and II, isolated from the digestive juice of a giant African snail, *Achatina balteata*, have been purified to homogeneity as demonstrated by the usual criteria of purity [4]. These extracellular enzymes are highly specific for the β-anomeric configuration of the glycosidic linkage.

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They catalyse the hydrolysis of various derivatives of β-D-galactose, β-D-fucose and β-D-glucose, but the values of kinetic parameters indicate that catalytic efficiency is maximum towards β-D-fucosides [5]. To our knowledge, these enzymes are the first β-glycosidases to be found to possess such a β-D-fucosidase activity. In a recent paper their main molecular and physicochemical properties have been studied [6]. As a first step in the elucidation of the mechanism of action of β-fucosidase, the identification of amino acid functional groups directly or indirectly involved in the mechanism of catalysis has been undertaken. This report presents data concerning β-fucosidase (form II) which possesses a monomeric structure with a molecular weight of about 110 000 [6].

Purification of β-fucosidase and enzyme assays were carried out as described previously [4]. Specific activity of purified enzyme, using *p*-nitrophenyl-β-D-fucoside (Sigma) as substrate, was 63 μmol liberated aglycone/min per mg at 37°C and pH 5.4 [5].

Reaction of iodination of enzyme was performed at pH 5.4 and 25°C with different concentrations of iodine as potassium iodide. Deactivation kinetics were also studied as a function of time. The results shown in Fig. 1 indicate that the enzyme was markedly inactivated by a 60-fold molar excess of iodine. The iodination of proteins may essentially affect sulfhydryl or tyrosine residues or both. However, since thiol-specific reagents (Table I) failed to inhibit the activity and since the iodine inhibition could not be reversed by cysteine, there is no need to involve thiol groups in the inhibitory effect of iodination. Moreover, the enzyme could be protected completely against inactivation when the reaction was performed in the presence of lactose (Fig. 1) which is a substrate for β-fucosidase [5]. These first observations suggest that the loss of activity by iodine is due to the modification of residues at the active site.

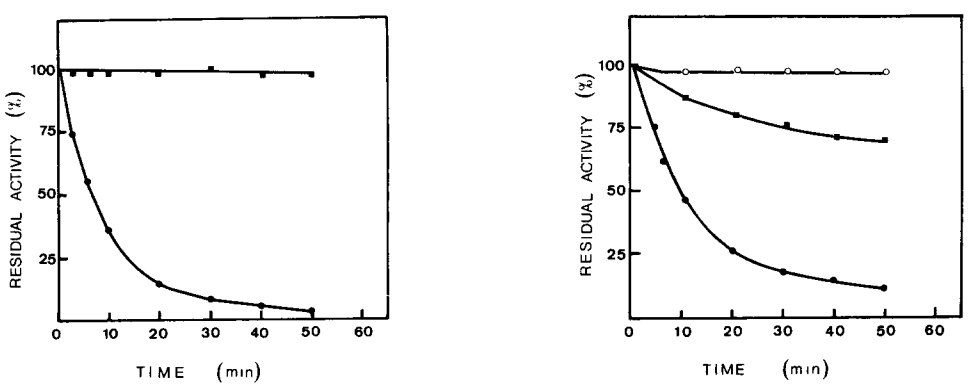

Fig. 1. Effect of iodination with 60-fold molar excess of iodine on the activity of β-fucosidase. The reactions were performed at 25°C in 100 mM acetate buffer, pH 5.4 (●———●). For the protection experiments (■———■), lactose (100 mM) was added before iodine. Aliquots were withdrawn from the reaction mixture at the indicated times and after convenient dilution assayed for enzyme activity.

Fig. 2. Effect of nitration with 100-fold molar excess of tetranitromethane on the activity of β-fucosidase. The reactions were performed at 25°C in 100 mM acetate buffer, pH 5.4 (○———○) and 100 mM phosphate buffer pH 8.0 (●———●). For the protection experiments (■———■) at pH 8.0, lactose (100 mM) was added before tetranitromethane. Aliquots were withdrawn from the reaction mixture at the indicated times and after convenient dilution assayed for the enzyme activity.

TABLE I

EFFECT OF THIOL-SPECIFIC REAGENTS ON ENZYMATIC ACTIVITY OF β-FUCOSIDASE

The enzyme was incubated for 60 min at 25°C with the reagents indicated. Aliquots were withdrawn and after dilution, enzymic activity was determined and expressed as a percentage of the initial activity.

Reagent	Concentration (mM)	Residual activity (%)
N-Ethylmaleimide	100	94
Iodoacetamide	50	97
Iodoacetate	50	96

Although iodination concerns mainly tyrosine and sulfhydryl groups, possible reactions with tryptophan, histidine and methionine residues of protein cannot be entirely ruled out. For this reason, another tyrosine-specific reagent, tetranitromethane, was used to determine whether the inactivation observed with iodine might be due to reaction at a site other than a tyrosine residue. This chemical affects only tyrosine and sulfhydryl residues. Besides, according to Sokolovsky et al. [7], nitration of tyrosine residues does not proceed below pH 7.0, while sulfhydryl groups continue to be oxidized. Tetranitromethane (Sigma) was diluted 10-fold with 95% ethanol before further dilution into the reaction mixture [7]. Nitration of enzyme was carried out in 100 mM phosphate buffer at pH 8.0 and 25°C. As shown in Fig. 2, nitration of β-fucosidase caused profound loss of activity. When a substrate of the enzyme such as lactose was added before tetranitromethane, the activity fell to only 70% the initial activity. This protection against inactivation was not complete as in the case of iodination. But, it is to be noted that treatment with tetranitromethane was performed at pH 8.0. In these conditions the affinity of lactose for β-fucosidase is very low and consequently the protection of the active site is less effective. Experiments were also carried out at pH 5.4 in 100 mM acetate buffer. No loss of activity was observed in this case. Again, these findings are consistent with the presence of essential tyrosine residues.

In order to verify that tyrosine residue modification was well implicated in the inhibition process of β-fucosidase, the protein was treated with *N*-acetylimidazole (Sigma), another specific reagent of tyrosine residues. Acetylation was performed at 25°C in 50 mM sodium barbital buffer (pH 7.5), using a 120-fold molar excess of *N*-acetylimidazole. A progressive inactivation of catalytic activity was observed. After 60 min of incubation, a loss of 80% activity was obtained. Treatment of the modified enzyme using 0.5 M hydroxylamine, restored the enzymic activity which approximated to that of the native enzyme treated in the same conditions as control.

In conclusion, the most striking feature of our results is the identical effect of iodination, nitration and acetylation on the activity of β-fucosidase and the effective protection against inactivation by a substrate. These observations provide evidence for the presence of functional tyrosine residues at the substrate binding site of β-fucosidase.

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