α- AND β-GALACTOSIDASES BOUND TO NYLON NETS

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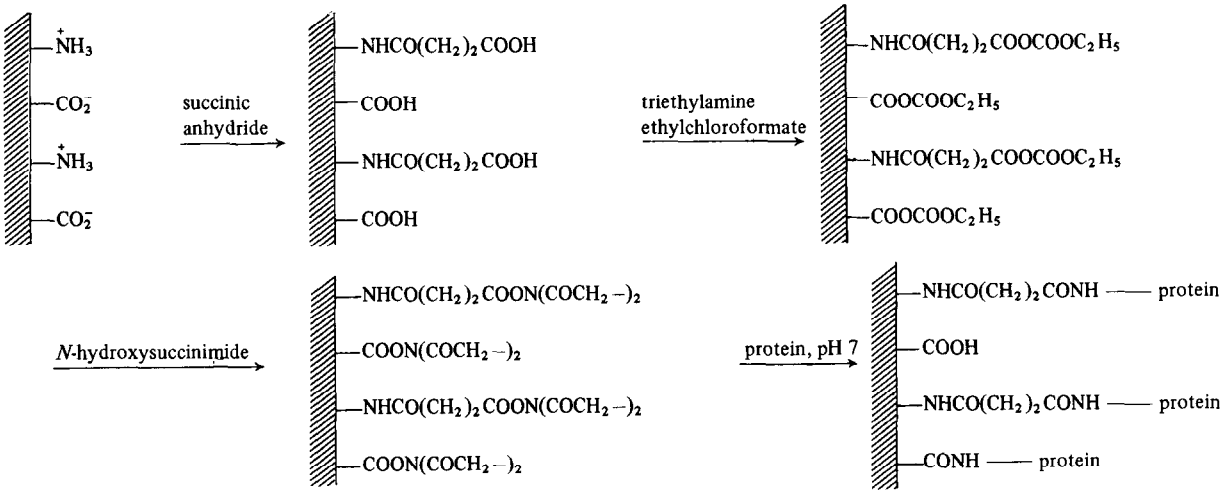
1. Introduction

In the last years different supports have been used for the immobilisation of β-galactosidase. Kay et al. [1] attached the enzyme to cellulose supports, Robinson et al. [2] and Woychik and Wondolowski [3] to glass surfaces. Hustad et al. [4] used a polyisocyanate polymere coat on Teflon, and only recently Inman and Hornby [5] covalently attached β-galactosidase to the inside surfaces of nylon tybes. Up to date nylon has only rarely been used as a solid support, mainly by Hornby et al. [5–7] and Allison et al. [8]. Biospecific separation of cells by surface reaction has been successfully performed by Edelman et al. [9] on fibres coated with lectins or antibodies. In one case the proteins were covalently attached to the partially hydrolysed nylon surface by means of a soluble carbodiimide. A more gentle coupling reaction was used by Hornby et al. [5–7] and Allison et al. [8] with glutaraldehyde.

In our experiments we covalently attached proteins to nylon surfaces making use of our experience in peptide chemistry. After partial hydrolysis of nylon we converted the free amino groups on the surface to carboxylic groups by succinylation. After thorough drying the carboxylic groups were activated via mixed carbonic anhydrides, according to Wieland [10], Boissonas [11], and Vaughan [12] and without isolation converted to the activated *N*-hydroxysuccinimidesters. Introduced by Anderson [13], these esters have been successfully used by Cuatrecasas and Parikh [14] for the preparation of agarose supports for affinity chromatography.

The hydroxysuccinimidester-activated nylon surfaces were reacted with antibodies [15] and various enzymes. Under the conditions of the coupling reaction β-galactosidase retained its full enzymic velocity, and α-galactosidase about 40%.



2. Materials and methods

α-Galactosidase from green coffee beans and β-galactosidase from *E. coli* were cristalline suspensions (5 mg protein/ml) from Boehringer, Mannheim. *p*-Nitrophenyl-α-D-galactopyranoside and *p*-nitrophenyl-β-D-galactopyranoside were purest grade from Serva, Heidelberg. Methyl-β-D-galactopyranoside [^{14}C]methyl with a specific activity of 3 mCi/mMol was from NEN, Dreieichenhein, the unlabelled compound, pure state, from Koch Light Labs, Bucks, England.

2.1. *Superficial hydrolysis and succinylation of nylon*

Nylon nets (10 ccm × 10 cm), were washed for 10 min both in petroleum ether (b.p. 30–40°C) and tetrachloromethane, and hydrolysed in a Petri dish at 25°C in 4.52 N hydrochloric acid for 30 min [9]. The nets were washed free from acid with water and dried in vacuo. In some cases the hydrochloric acid and the washings were combined and evaporated to dryness in order to determine the nitrogen content according to Kjeldahl. About 100 cm^2 of the hydrolysed nylon nets was succinylated [16] in 200 ml of 0.1 M K_2HPO_4 at pH 9 by adding 4 g of solid succinic anhydride and, under stirring, keeping the pH 9 by dropwise addition of 1 M NaOH until all of the anhydride was dissolved. After 4 hr reaction the net was washed with 500 ml 50% acetic acid, several times with water, with 200 ml each of 1,4-dioxane and dimethylformanide, and dried in vacuo.

2.2. *N-Hydroxysuccinimidesters of nylon and coupling of proteins*

100 cm^2 of the dry succinylated nylon was put into 200 ml of amine free anhydrous dimethylformamide. 3.48 ml of triethylamine and 2.4 ml ethylchloroformate were added at −15°C to the stirred suspension. After 15 min reaction the net was transferred to a beaker with 3 g of *N*-hydroxysuccinimide dissolved in 100 ml in dimethylformamide. The net was stirred for 70 min at 25°C, rapidly washed with ice water, and transferred to the solution of the protein to be coupled, either in 0.9% NaCl or in 0.1 M buffers of pH 6.5 to 8.4. A homogenous attachment of the proteins to the nylon surfaces was achieved by shaking activated nets in the protein solutions for 10 hr at 25°C. In order to bind the galactosidases 25 cm^2 of activated nylon net was vigourously shaken in 20 ml of KH_2PO_4 buffer, pH 6.5, with 1 mg of the enzymes for 12 hr. After several washings the immobilized enzymes were stored in the same buffer at 4°C.

2.3. *Enzyme assays*

All assays were performed in 0.1 M KH_2PO_4 buffer, pH 6.5. α-Galactosidase activity was determined by vigourously shaking on a rotation mixer 1 ml volumes of a buffer solution of 3.2×10^{-5} M to 1.6×10^{-2} M *p*-nitrophenyl-α-D-galactopyranoside at 25°C, together with 0.25 cm^2 of the nylon-α-galactosidase net, corresponding to 1 μg of protein. After 10 min the nets were taken out, 2 ml of a 0.2 M borate buffer pH 9.8 were added and the optical density was measured at 405 nm. About 20% of the *p*-nitrophenol liberated remained adsorbed to the nylon net and was estimated after extraction of the nets with 2.5 ml of dimethylformamide. β-Galactosidase activity was determined similarly with 0.125 cm^2 of the nylon-β-galactosidase net, corresponding to 0.7 μg of protein, in a 5 min reaction with substrate concentrations from 3.2×10^{-5} M to 3.2×10^{-2} M.

The kinetics of hydrolysis of methyl-β-D-galactopyranoside [^{14}C]methyl (specific activity 300 μCi/mM) was followed by shaking 25 ml of a 1.5×10^{-3} M solution in 0.1 M KH_2PO_4, pH 6.5, together with 8 cm^2 of the nylon-β-galactosidase net corresponding to 44.8 μg protein (A). A second run in parallel (B) contained free enzyme (20 μl of cristalline suspension/58 ml buffer, corresponding to 43.1 μg of protein/25 ml) instead of the nylon net. During 24 hr samples of 1 ml were taken from both reaction vessels and substituted by enzyme solution or buffer, respectively. The samples were evaporated, evaporated twice with methanol, dissolved in 1 ml water, and 200 μl samples were used for radioactivity determination.

3. Results and discussion

Hydrolysis of surfaces of nylon nets without destruction of the texture is possible with hydrochlorid acid in a narrow range around 4.52 N. The acid treatment caused shrinkage of the nylon by some 20% and released 8 mg *N*/g nylon after 30 min. After succinylation and activation as *N*-hydroxysuccinimidesters the binding capacity of the surfaces was determined by reaction with [^{14}C](N^{ind}-methyl)-secophalloidin [17],

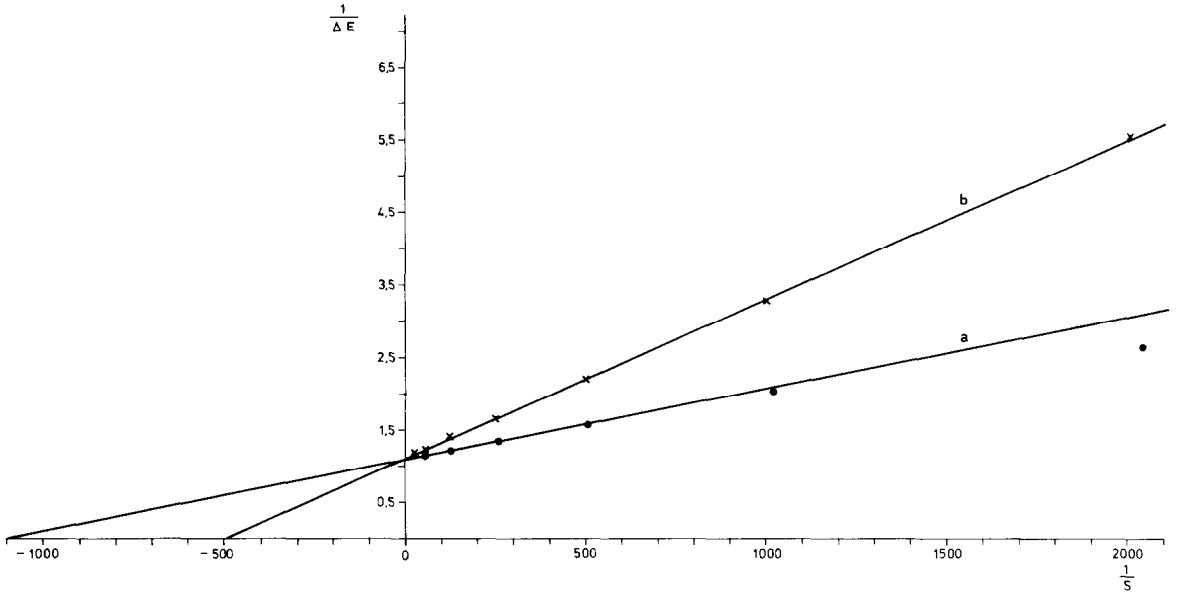


Fig. 1. Lineweaver-Burk plot of the hydrolysis of *p*-nitrophenyl-β-D-galactoside with β-D-galactosidase attached to nylon (a) and in solution (b).

a cyclic peptide of mol. wt. 803 with a free α-amino group, which was taken as a better model compound than a labelled amine of lower molecular weight. By this reaction the capacity of activated carboxyls was determined to be at least 0.1 $\mu M/cm^2$. In the following coupling reactions only 0.01% of all activated carboxyls reacted with the enzymes.

The coupling reaction of the two enzymes yielded 4 μg protein attached to 1 cm^2 of the nylon net in the case of α-galactosidase, and 5.6 $\mu g/cm^2$ in the case of β-galactosidase. The *N*-hydroxysuccinimide released in the reaction heavily affected the protein assay of Lowry [18]. Therefore the coupling of the protein was followed by measuring the rest activity of the enzyme in the reaction medium after removal of the nylon nets. When 40 μg of protein were reacted with 1 cm^2 of activated nylon net, usually 10–16% were attached to the nylon surface.

For comparison of the immobilized and free enzymes the specific activities in the hydrolysis of the *p*-nitrophenylgalactosides were measured. In the case of α-D-galactosidase the specific activity was 10.8 U/mg for the free enzyme and 4.1 U/mg in the nylon attached state. Thus the specific activity was decreased to 38%. In the case of β-D-galactosidase the specific activity was 19.1 U/mg in both states. This means that the enzymatic function had not been affected anyway by the attachment to the nylon surface. This result was confirmed by a Lineweaver-Burk plot (fig. 1),

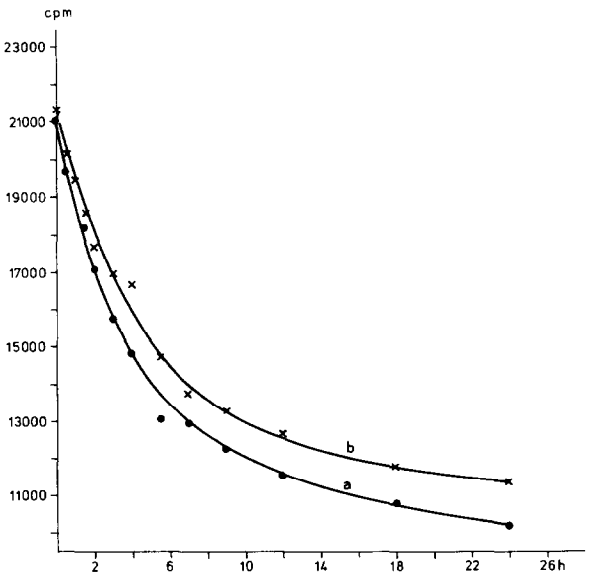


Fig. 2. Hydrolysis of methyl-β-D-galactoside with β-D-galactosidase linked to nylon (a) and in solution (b).

which gave identical V_{max} values for the enzyme in solution and the enzyme on the nylon surface. The K_m-values, however, differed by the factor 2.2. They were 0.9×10^{-3} M for the free and 2.0×10^{-3} M for the insolubilized enzyme. Similar effects on the K_m-values had been observed by Hustad et al. [19] for β-galactosidase insolubilized on a Teflon coated stirring bar with lactose as substrate. The values in this case were 1.31×10^{-2} M and 2.21×10^{-2} M for the free and the immobilized enzyme, respectively. Evidently the formation of the enzyme–substrate complex is partially inhibited for the nylon attached enzyme; the inhibition appears in the Lineweaver-Burk plot to be of a competitive type.

In another, more realistic experiment we followed the hydrolysis of methyl-β-galactoside, a nonactivated substrate at a concentration of 1.5×10^{-3} M, which is far below the K_m-value of this substance. Fig. 2 shows that the reaction rate of the nylon bound enzyme was similar to that of the free enzyme, differing by only 10% after 24 hr of incubation.

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