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# INFLUENCE OF ELECTROSTATIC INTERACTIONS AND ENZYME REACTIVITY ON THE BINDING MODE OF ALPHA-AMYLASE

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ABSTRACT

Alpha-amylase was coupled to cellulose and carboxymethylcellulose through activation with cyanogen bromide. The specific activities, retentions of native enzyme activity, and action patterns of the resulting immobilised derivatives depended markedly on the pH of the coupling reaction. This dependency is discussed in terms of the role of the coupling pH in the regulation of electrostatic interactions and enzyme reactivity.

## INTRODUCTION

Attachment to insoluble carriers usually causes a considerable loss in the catalytic efficiency of enzyme molecules[1]. This effect is generally attributed to such factors as conformational changes in the three-dimensional structure of the enzyme[2], steric hindrance caused by matrix elements[3], and resistance to external and internal diffusion[4]. Examples have been reported in which enzymes are totally inactivated on immobilisation, probably because of exclusion of the bulky substrate molecules from the porous carrier network where the enzyme is located[5], or to drastic physical or chemical alterations in the active site[6]. Inactivation due to restraint of internal diffusion may occur even with substrates of small molecular size. Thus, Weibel and Bright[7] found that when D-glucose oxidase was attached to porous glass particles, only a small fraction (6%) of the bound enzyme, namely, that on the surface, was active. The problem becomes more serious with polymeric substrates, for example, starch fractions, the molecular weight of which can be as high[8] as $10^7$–$10^8$.

Thus, selective attachment of an enzyme to the surface of a carrier is important, and the hypothesis that increasing the reactivities of the components in the coupling reaction results in increased surface-binding[9] provides a means to predict and control the extent of surface binding in enzyme immobilisation. The binding mode of alpha-amylase accords[10] with the hypothesis, and we now report on the effect of the coupling pH on the binding mode of this enzyme.

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EXPERIMENTAL

*Materials.* — Alpha-amylase was obtained from Novo (type BAN 1000 S). The specific activity of this enzyme is reported[10] as 27540 U/g, as measured under the conditions described below. Carboxymethylcellulose (type CM 52) was obtained from Whatman. Native amylose (number-average molecular weight, 650,000) was obtained by fractionation[9] of potato starch. The exclusion limit of CM 52 was estimated[11] to be $>10^5$. The number-average molecular weight of soluble starch was reported[12] as $3.0–3.5 \times 10^4$.

*Activation of the carrier.* — Cellulose and CM 52 were activated essentially by the method of Porath *et al.*[13]. Portions (equivalent to 50 mg of dry polymer) of carrier were dispersed in 3M phosphate buffer (pH 11.0), equilibrated, and mixed with CNBr (60 mg) in a reaction volume of 7 mL. The procedure was described in detail elsewhere[9,10].

*Coupling of alpha-amylase.* — Solutions of alpha-amylase were prepared in either 0.25M acetate (pH 4.0, 4.5, 4.8, 5.0, 5.1, 5.4, 5.5), 0.25M boric acid–acetate (pH 6.5), or 0.25M boric acid–sodium hydroxide (pH 9.0, 10.5). These buffers do not contain strong sequestering agents for calcium which would be detrimental to the stability of the enzyme[14], or nucleophilic groups that would compete with the enzyme for the reactive groups on the carrier; 0.25M buffers were of adequate strength for the intended use. BAN 1000 S (90 mg) was added to the coupling medium containing the carrier (50 mg, dry basis) immediately after activation, the volume was made up to 5 mL with the relevant buffer, and the mixture was stirred for 20 h at 4°. The mixture was then decanted, and the residue was washed 7–8 times, alternately, with cold boric acid–acetate (pH 6.5) and boric acid–acetate–sodium hydroxide (pH 8.5) buffers, and finally with cold water. A blank prepared in the same way, except for the exclusion of CNBr, showed no activity during prolonged exposure to soluble starch.

The amount of bound enzyme was found by subtracting the amount present in the decantate and combined washings from the amount added to the coupling medium. Protein concentrations were determined as described by Lowry *et al.*[15].

*Enzyme assays.* — Activities of soluble and insoluble enzymes were assayed by measuring the amount of reducing sugars released during 10 min at 30° by the action on 0.5% soluble starch in 0.05M acetate buffer (pH 6.0). Reducing sugar was determined as described by Nelson[16], and expressed as glucose equivalents. Accordingly, 1 unit (U) is equal to the amount of catalyst that liberates 1 $\mu$mol of glucose equivalent per min from soluble starch under the specified conditions. The procedure was given in detail elsewhere[9].

Specific activities of the insoluble derivatives were expressed as units per g of dry carrier. The percentage retention of original activity was found by dividing the specific activity of the immobilised enzyme by the solution activity of the protein covalently bound to 1 g of dry carrier. Figures thus obtained allow an assessment of inactivation due to immobilisation.

*Determination of action patterns.* — Alterations in the action patterns of native and insoluble alpha-amylases were determined from plots of iodine blue-value *versus* reducing sugar[17]. Aqueous 0.2% amylose (100 mL) at 40° was mixed with suitable amounts of catalyst. Aliquots (4 mL) were withdrawn from the continuously stirred reaction mixture and added immediately to an equal volume of 0.1M HCl. The resulting mixture was stable for at least 2 days as confirmed by reducing sugar[16], blue value[18], and glucose[19] determinations. Samples collected during 2–8 h were centrifuged at 3200 r.p.m. for 10 min and decanted. Reducing[16] and blue values[18] were determined.

## RESULTS AND DISCUSSION

The effect of the coupling pH on the specific activity of the resulting immobilised alpha-amylase is shown in Fig. 1. A tri-phasic behaviour was observed for the enzyme coupled to carboxymethylcellulose, with a distinct minimum at pH 5.4 which is the isoelectric point of the enzyme[20]. At pH >8.0, the amount of enzyme bound fell continuously with increase in pH, probably because of hydrolysis of the imido carbonate groups on the carrier. At pH 8.0, 198 mg of BAN 1000 S was bound per g of dry carrier, whereas the corresponding value at pH 10.5 was 60 mg.

At pH >3, CM 52 is negatively charged; at pH 4.5, ~90% of the carboxyl groups are deprotonated[21]. Since the enzyme carries a net positive charge below pH 5.4 (the isoelectric point), there are favorable electrostatic interactions for couplings below pH 5.4; the amounts of enzyme bound at pH 4.5 and 5.4 were 116 and 50 mg per g of dry carrier, respectively. That higher amounts of bound enzyme and

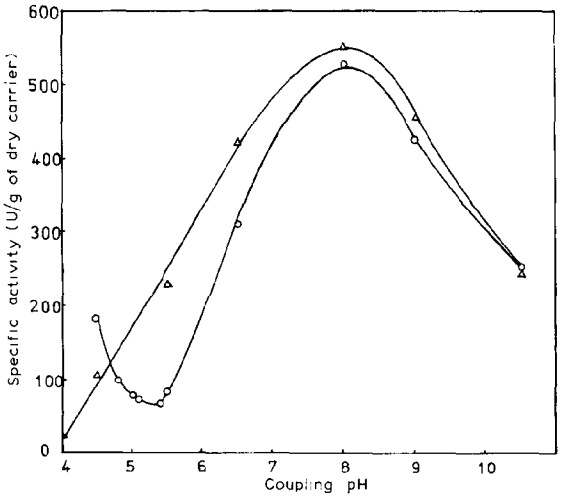


Fig. 1. Effect of the coupling pH on the specific activity of immobilised alpha-amylase: △, cellulose-based derivatives; ○, CM 52-based derivatives.

higher specific activities are achieved in coupling reactions where the enzyme and the carrier are oppositely charged has been reported[22,23], although it is not clear whether the increased specific activity was due to increased loading or to a change in the binding mode.

Fig. 2 depicts the effect of the coupling pH on the retention of enzyme activity. The curve for the carboxymethylcellulose–enzyme derivative passes through a minimum at pH 5.4, whereas a more or less uniform behaviour is observed for the cellulose–enzyme derivative. If the variation in the stability of the native enzyme with pH is taken into account (Table I), the minimum at pH 5.4 is more pronounced than displayed in the curve. The differences between the two curves are probably due to changes in the binding mode of the enzyme. Below pH 5.4, electrostatic interactions between the enzyme and carboxymethylcellulose are intensified as the coupling pH is lowered, and the rate of the coupling reaction is increased which results in a higher proportion of binding in the initial phases of the coupling reaction. It is also conceivable that electrostatic interactions are less influential on the surface than inside the pores where the mobility of the enzyme molecules is likely to be restricted. This would increase the probability of binding within the pores and result in their early filling, so that subsequent bindings would be limited to the exterior surface of the carrier. The operation of this selectivity depends on the pore size of the carrier and the molecular weight of the enzyme[10]. Alteration in the binding mode of the enzyme is manifested in the degree of retention of activity, since accessibility of the enzyme for the relatively bulky molecules

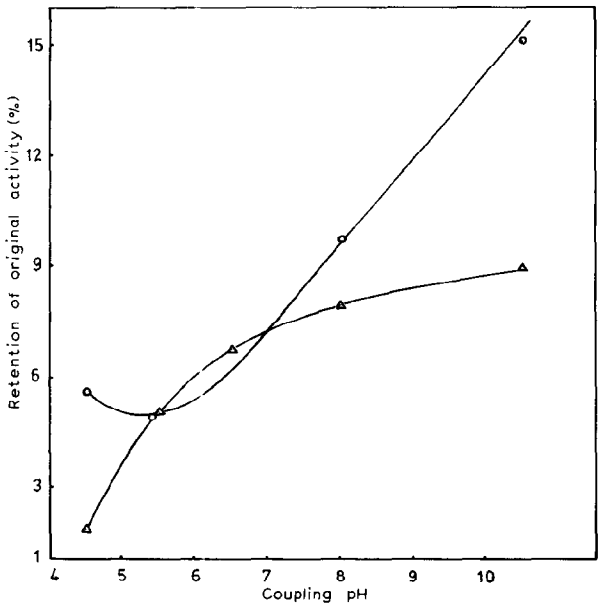


Fig. 2. Effect of the coupling pH on the retention of native enzyme activities. Data correspond to those in Fig. 1.

TABLE I

STABILITY OF NATIVE ALPHA-AMYLASE[a] AT DIFFERENT pH VALUES

| pH | 4.5 | 4.8 | 5.1 | 5.4 | 6.5 | 8.0 | 9.0 | 10.5 |
|---|---|---|---|---|---|---|---|---|
| Residual activity (% of initial value) | 27.6 | 62.1 | 68.1 | 84.5 | 93.3 | 100.0 | 100.0 | 100.0 |

[a]BAN 1000 S (90 mg/mL) at 4° for 1 week.

of soluble starch is increased when couplings occur preferentially on the surface of the carrier.

At higher pH values, enzyme reactivity is increased because of an exponential rise in the concentration of unprotonated amino groups through which the reaction proceeds. The increase in the number of reactive centres on the enzyme molecule increases the probability of multi-point attachments giving rise to bound enzymes that are probably less mobile and more chemically strained. Since this would result in a higher degree of inactivation of the enzyme, retention of activity should be inversely proportional to the enzyme reactivity during coupling. However, curves displayed in Fig. 2 indicate that the retention of activity increases with enzyme reactivity; the initial decline in the curve for CM 52 corresponds to the region of decreasing enzyme reactivity due to charge neutralisation as the pH is increased from 4.5 up to the isoelectric point, 5.4. Although the amount of bound enzyme decreases as the coupling pH is raised above 8.0 (Fig. 1), because of hydrolysis of the reactive groups on the carrier, the activity of the bound enzyme relative to that of the soluble enzyme keeps increasing (Fig. 2). This is taken to be an indication of the change in the binding mode of the enzyme. As enzyme reactivity during coupling is increased, the proportion of the enzyme bound onto the outer surface of the carrier increases. This gives rise to a more efficient use of the total bound enzyme, since a larger portion of it is made accessible to bulky substrate molecules.

However, this may not be the sole effect produced by increasing the coupling pH. Some variation may be expected in the types of nucleophilic groups of the enzyme molecule which are likely to take part in the reaction as the pH is varied. Although it has been reported that the reaction proceeds, at least predominantly, through the free amino groups of the ligand protein[24], it is conceivable that, especially when the coupling pH is low, histidine groups (with lower $pK_a$ values) are also involved in the reaction. Lack of information on the structure of the active site of *Bacillus subtilis* alpha-amylase makes it difficult to predict the precise outcome of such a shift in the binding sites. Nevertheless, judging from the behaviour of the curve drawn for cellulose-based catalysts in Fig. 2, it can be inferred that any change in the degree of inactivation by damage to the active site, as the coupling pH is varied, is either insignificant or is masked by the predominant influence of the binding mode on the retention of activity.

Blue value–reducing sugar curves are shown in Fig. 3. Immobilisation of the

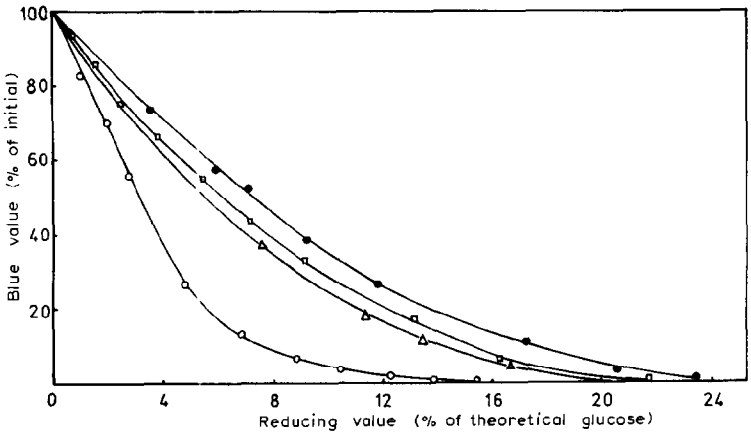


Fig. 3. Plots of blue value *versus* reducing value. Reducing values of hydrolysates are expressed as percentages of the values that can theoretically be reached: ○, native enzyme; □, coupling at pH 5.4; △, coupling at pH 8.0; ●, coupling at pH 10.5.

enzyme results in a change in the action pattern toward what has been described as multiple attack[25] or exo-enzymic behaviour[26]. The blue value is sensitive to changes in the distribution of molecular weight only if they involve significant modification in the molecular weight of species of high molecular weight[12]. The reducing value, on the other hand, is a colligative property and directly proportional to number-average molecular weight, a property that is more sensitive to changes in the concentration of species of low molecular weight. Thus, the shift from native enzyme behaviour observed in the curves for immobilised alpha-amylases indicates that, at a given reducing value (which corresponds, in all cases, to a given number of catalytic cleavages), the action of immobilised enzymes produces smaller modifications in the large molecules. This picture could result either from an exo-enzymic action or from a preferential attack on smaller substrate molecules, or from both. In fact, immobilised alpha-amylases, as opposed to soluble alpha-amylases, hydrolyse smaller molecules faster than long-chain polysaccharides[9]. This alteration in behaviour originates from limitations due to intraparticle diffusion and steric hindrance, which make the smaller molecules more competitive than larger ones with respect to access to the catalytic centers. The largest deviation from the native enzyme behaviour is observed for the catalyst prepared at pH 10.5 which probably has the highest density of cross-links.

Thus, the coupling pH has a profound effect not only on the amount of enzyme bound but also on its catalytic efficiency. For such a charged carrier as CM 52, the pH of the coupling reaction regulates the nature and intensity of electrostatic interactions in addition to the reactivities of the nucleophilic centers on the enzyme molecule. It appears that this regulatory function can be used to advantage in optimising the use of enzymes in immobilisation.

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