# THE ACTION PATTERN OF WATER-INSOLUBLE α-AMYLASES

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## 1. Introduction

α-Amylase (EC 3.2.1.1.) catalyses the hydrolysis of amylaceous polymers producing small branched oligosaccharides together with variable amounts of glucose, maltose and maltotriose depending on the source of the enzyme. It has long been established [1] that differences in the action patterns of α-amylases from different sources can be observed by plotting the "blue value" (i.e. the intensity of iodine staining) against the reducing power during the course of α-amylolysis. The term "multiplicity of attack" is used to describe the number of catalytic events (i.e. α-1,4 glucosidic links hydrolysed) which occur on a single amylose chain during the life time of the enzyme-substrate complex. Since each hydrolytic event releases one reducing group and the blue value is an inverse function of the amylose chain length it follows that an α-amylase exhibiting a high degree of multiple attack will result in a greater increase in reducing power for a given decrement in blue value than an α-amylase exhibiting a lower degree of multiple attack.

In the present work the action pattern of three water-insoluble derivatives of α-amylase has been investigated. In each case it has been found that these products exhibit a higher degree of multiple attack than the water-soluble enzyme from which they were prepared. We believe this to be the first report of a change in the nature of the products as a result of the coupling of an enzyme to an insoluble support.

## 2. Experimental

Crystalline bacterial α-amylase (type II-A, ex. B. subtilis, obtained from Sigma Chemical Co., London) was coupled to CM-cellulose and p-aminobenzyl-cellulose according to the methods of Mitz and Summaria [2] and Campbell, Leuscher and Lerman [3] respectively and to polystyrene according to the method of Grubhofer and Schleith [4]. All insoluble α-amylase preparations were stored at 4°C as water suspensions. Chemically attached protein was determined according to Hornby, Lilly and Crook [5]. α-Amylolysis was allowed to proceed at 25°C in 0.1% (w/v) solutions of amylose (British Drug Houses), 0.1M in phosphate buffer pH 5.8, with rapid stirring. The blue value was estimated at intervals by taking 2 ml aliquots of reaction mixture, adding 2 ml if iodine-potassium iodide solution,

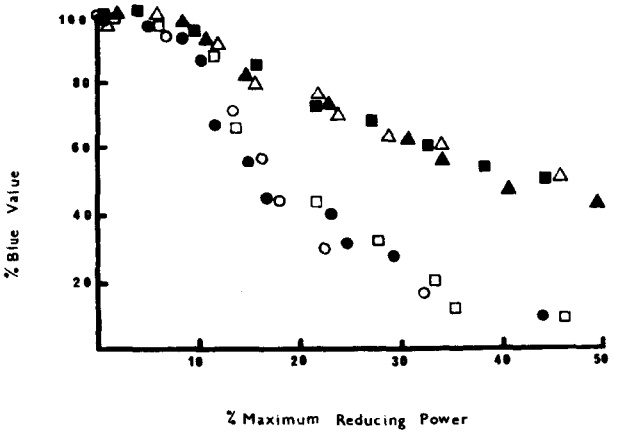


Fig. 1. Plots of blue value against reducing power for the hydrolysis of amylose by water-insoluble derivatives of α-amylase in a continuous stirred reactor and for free α-amylase in solution. (▲) CL-cellulose-α-amylase; (△) p-aminobenzyl-cellulose-α-amylase; (■) polystyrene-α-amylase; (●) soluble α-amylase, 10 μg per ml; (□) soluble α-amylase, 500 μg per ml; (○) 1000 μg per ml.



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Table 1
Protein content and enzymic activity of water-insoluble α-amylases

| Preparation | % Bound protein (w/w) | Specific activity (units* per mg. of protein) |
|---|---|---|
| Free α-amylase | – | 820 |
| CM-cellulose-α-amylase | 2.1 | 35 |
| PAB-cellulose-α-amylase | 1.5 | 40 |
| Polystyrene-α-amylase | 4.0 | 75 |

* One unit of α-amylase activity will liberate one mg of maltose from soluble starch in 3 min at pH 6.9 at 20°C.

diluting to 50 ml with water and reading the optical at 610 mμ. Reducing sugar was estimated at the same time intervals according to Hodge and Hofreiter [6]. Glucose was estimated using the glucose oxidase-peroxidase enzymatic assay coupled to the chromogenic acceptor 0-dianisidine [7] (Sigma Kit No. 510, Sigma Chemical Co., London).

## 3. Results and discussion

The protein content and α-amylase activity of the three water-insoluble enzyme preparations are shown in table 1.

The plots of the blue value against the reducing power of the assay mixture as α-amylolysis proceeds reveal that each of the insoluble derivatives, when assayed in a continuous stirred reactor, exhibited a higher degree of multiple attack than the free enzyme in solution (fig. 1). The results shown for the free enzyme were obtained from experiments in which the concentration of free enzyme varied from 10 to 1000 μg per ml of reaction mixture. There is no significant trend towards higher degrees of multiple attack within this 100-fold concentration range of free enzyme. Therefore, it seems unlikely that the higher degree of multiple attack shown by the water-insoluble preparations is due to any localised concentration of bound enzyme on the surface of the support.

The possibility that diffusion of the substrate to

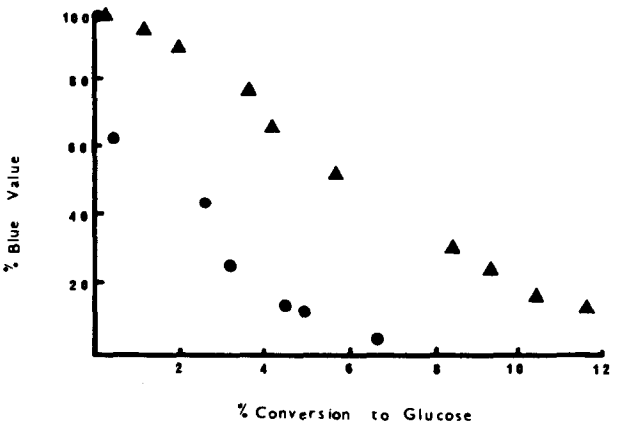


Fig. 2. Plots of blue value against glucose for the hydrolysis of amylose by polystyrene-α-amylase in a continuous stirred reactor (▲) and soluble α-amylase in solution (●).

the surface of the bound enzyme can contribute to the elevated degree of multiple attack shown by the insoluble derivatives was investigated by employing these preparations in the form of packed beds. In this experiment, the substrate was pumped from a reservoir, through the column and back to the reservoir in a continuous cycle, samples for analysis being taken from the reservoir as α-amylolysis was proceeding. In this way action patterns were obtained at flow rates of 1.5, 5 and 10 ml/min through the column. The results of this experiment revealed no significant difference between the action patterns obtained at the three different rates of substrate perfusion. Since the rate of equilibration of substrate between the bulk of the substrate solution and the surface of the bound enzyme is inversely related to the rate of perfusion through the column [8] it therefore follows that any limitation in the rate of substrate diffusion cannot contribute to the elevated degree of multiple attack of the bound enzymes.

To investigate the nature of the enhanced multiplicity of attack of the coupled α-amylases, the production of glucose, normally only a minor product of α-amylase action, was studied as a function of decreasing blue value for both free α-amylase and polystyrene-α-amylase. The results show marked increase in the production of free glucose when the enzyme is attached to the polystyrene (fig. 2). The possibility exists that this increased production of

glucose is the outcome of preferential concentration onto the polystyrene of any α-glucosidase contaminant in the original commercial enzyme preparation. Against this is the observation that repeated coupling from the same solution of α-amylase (possible because of the small percentage of enzyme coupled) to six separate batches of polystyrene gave products that revealed no difference in plots of blue value against glucose.

The most likely explanation of the increase in multiplicity of attack is that it is a steric effect arising from the particulate nature of the support. Such effects have been previously suggested [5] for the plant protease ficin attached to CM-cellulose, where the specific activity of the bound ficin relative to the free enzyme was 12% and 4% when the substrates were N-α-benzoyl-L-arginine ethyl ester and casein respectively.

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