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CHARACTERIZATION OF CELLULASE IMMOBILIZED ON DEXTRAN AND DIETHYLAMINOETHYL-DEXTRAN

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ABSTRACT

Cellulase was covalently bound to water-soluble dextran, by means of 2-amino-4,6-dichloro-s-triazine, and immobilized on diethylaminoethyl (DEAE)-dextran via ionic linkage at pH 4.0. The free enzyme had a maximal specific activity, using carboxymethyl-cellulose as substrate, at pH 5.0 while the dextran-bound and DEAE-dextran-bound enzymes had maximal activites at pH 7.5 and 4.0, respectively. The free enzyme was most active at 50°C. Dextran-bound and DEAE-dextran-bound cellulase were most active at 55 and 60°C, respectively.

INTRODUCTION

Cellulase, an enzyme complex catalyzing the hydrolysis of cellulose into glucose, has attracted worldwide research interest and has been the subject of several technical symposia (1, 2, 3, 4, 5). The economic importance of this enzyme is traceable to the vast amount of photosynthesized cellulose which is annually produced, i.e., 10^{11} tons per year (6). The optimal utilization of cellulose for the production of food, fuel or industrial chemicals relies on the development of the technology for cellulase production and enzymatic saccharification of cellulose.

The cost of cellulase-based technology can be reduced by increasing enzyme activity and thermal stability. This may be accomplished by enzyme immobilization on suitable carriers. Previous work on the immobilization of cellulase was reported by Manecke (7) who observed that after five conversions and 10 days storage, 80% of the original activity of the resin-bound enzyme was retained. The adsorption of cellulase on cellulose has been studied and was found to increase the rate of cellulose hydrolysis (8, 9, 10). Although enzyme adsorption on the substrate could provide a simple method of separating the product glucose from both enzyme and cellulose, the extent of adsorption was found to decrease with increasing temperature for several types of cellulose (9). Enzyme immobilization on a soluble carrier, which increases the thermal stability of the enzyme and facilitates the latter's recovery, would be an attractive alternative and is worthy of investigation.

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The present paper deals with studies on the immobilization of cellulase on two water-soluble carriers, namely dextran and diethylaminoethyl (DEAE)-dextran, via covalent and ionic linkages, respectively. The immobilized and free enzymes were compared in terms of their specific activities, using carboxymethyl-cellulose as substrate, at different values of pH and temperature. The molecular aspects of cellulase immobilization are discussed.

MATERIALS AND METHODS

Enzyme preparation and immobilization – Cellulase was obtained from culture filtrates of *Trichoderma viride* QM 9414. The latter was a gift from the U.S. Army Natick Development Center, Natick, Massachusetts, U.S.A. The method of enzyme preparation followed that of Vilela et al. (11) with minor modifications.

Enzyme attachment to soluble dextran, which has a molecular weight of about two million daltons (Sigma Chemical Co.), employed the bifunctional reagent 2-amino-4,6-dichloro-s-triazine using the procedures of Lilly and co-workers (12, 13). The dextran-bound enzyme was applied onto the top of a Sephadex G-100 column (2.2 cm x 66 cm) and eluted with 0.005M sodium borate buffer (pH 5.0) which was saturated with toluene in order to prevent bacterial growth. Fractions (3 ml) were collected at 28°C in the Buchler fraction collector. A sample of the unbound or free enzyme was run separately on the Sephadex column for comparison of elution volumes.

Prior to immobilization on DEAE-dextran, the enzyme was concentrated by ultrafiltration through an Amicon PM-10 membrane and then dialyzed continuously for 16 hours with 0.025M sodium citrate buffer (pH 5.0). The enzyme was then passed through a Sephadex G-150 column and the highly active fractions were pooled and concentrated via ultrafiltration. The enzyme was attached to DEAE dextran, whose average molecular weight was two million daltons (Sigma Chemical Co.), after swelling the carrier in 0.05M sodium citrate buffer at pH 4.0. This pH was chosen for attachment after trial runs showed that it was optimal. Free cellulase was separated from the immobilized enzyme by ultrafiltration on an Amicon XM-300 membrane.

Enzyme assay – Cellulase activity was measured according to the method of Mandels and Weber (14) using, as substrate, carboxymethyl (CM)-cellulose (sodium salt, Sigma Chemical Co.). The standard assay consisted of incubating 0.5 ml of the enzyme with the substrate at pH 5.0 and 50°C for one or two hours in an Eberbach water bath-shaker. Reducing sugar produced by the enzyme was determined (expressed as glucose) using either the method of Shaffer and Somogyi (15) or the dinitrosalicylic acid method (16). Enzyme which had been inactivated by boiling for 10 minutes served as control. One international unit (IU) of cellulase activity on CM-cellulose is defined as one micromole of glucose produced per minute under the assay conditions mentioned above. Measurements of pH were done with a Radiometer pH meter Model pHM 26. The protein content of the free and DEAE-dextran-bound enzymes were determined using the methods of Lowry (17) and Kjeldahl (15).

RESULTS AND DISCUSSION

The elution profile of cellulase (β-1,4-glucanase), both free and covalently bound to soluble dextran, is shown in Figure 1. The dextran-bound enzyme was eluted between 140 and 160 ml and its peak overlapped with that of blue dextran. This result is to be expected since both carrier dextran and marker blue dextran have approximately the same molecular weight (MW) of two million daltons. The MW values of two different endo β-1, 4-glucanases from *Trichoderma viride* have been reported by Berghem et al. (18) as 12,500 and 50,000 and would not significantly increase the MW of the carrier dextran. The elution volume of the free cellulase is approximately double that of the dextran-bound enzyme, as shown in Figure 1, and indicates that the unbound enzyme molecule is substantially smaller in size than the dextran-bound enzyme. The distinct separation of free and immobilized enzymes by gel filtration allowed the separate characterization of each enzyme form.

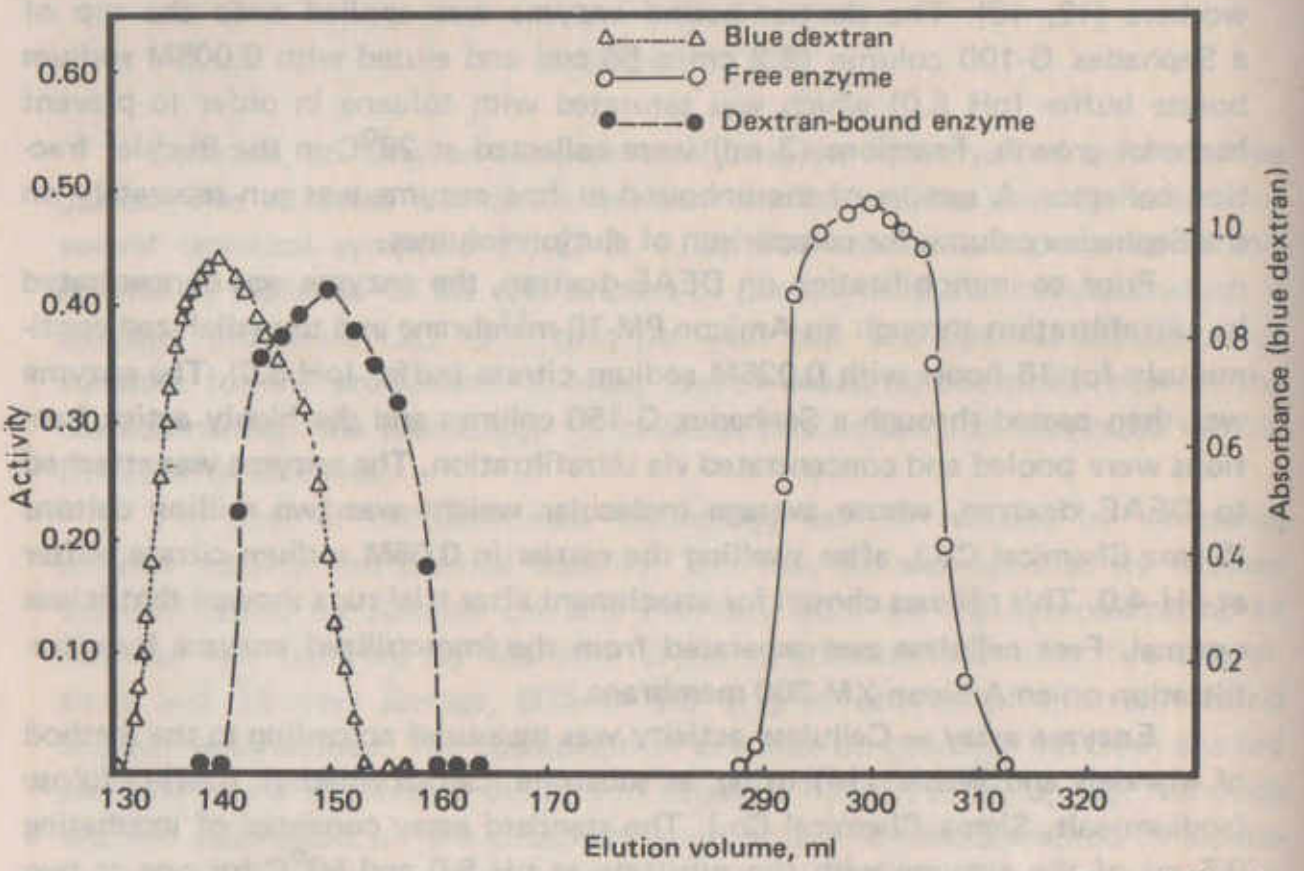

Figure 1. Chromatography on Sephadex G-100 of free and dextran-immobilized carboxymethyl-cellulase.

For the enzyme which was ionically bound to DEAE-dextran, ultrafiltration through an Amicon XM-300 membrane resulted in complete recovery of enzyme activity in the retentate despite exhaustive washing of the latter. No enzyme activity was observed in the ultrafiltrate. This indicates that all the enzyme molecules were bound to the DEAE-dextran carrier and were retained by the XM-300 membrane. The carrier loading factor was found to be 1.0 mg protein per gram DEAE-dextran. However, free DEAE-dextran molecules

were qualitatively detected in the ultrafiltrate. This should not be surprising, despite the reported protein molecular weight cut-off value of 3x105 for the XM-300 membrane, since dextran 110 (MW - 1.1×10^5) was not retained by the membrane (Amicon Publication No. 403).

The pH dependence of specific activity for free and immobilized CM-cellulase at 50°C is presented in Figure 2. The optimal pH for the free enzyme was found to be 5.0. This is similar to the optimal pH for the hydrolysis of CM-cellulose (pH 5.3) and cellulose (pH 4.2) by endoglucanase and cellotriose (pH 4.0) by exoglucanase from *T. viride* (19). Similarly, maximal hydrolysis was observed at pH 5.0 for alpha-cellulose and solka floc (14), modified cellulose (20) and rice straw (21) using *T. viride* enzyme. CM-cellulase immobilized on DEAE-dextran exhibited a shift in pH optimum towards a lower pH value. This pH shift is due to the polycationic property of DEAE-dextran which creates a charged microenvironment around the enzyme. This produces an unequal distribution of hydrogen and hydroxyl ions between the carrier phase and the external solution (22). The hydrogen ion concentration in the domain of the positively charged carrier is less than the concentration in the external solution, which is the phase measured by the pH meter (23). Consequently, the immobilized enzyme senses a pH which is higher than the external pH. Its pH optimum is, thus, shifted towards a lower pH value since more hydrogen ions should be added in the external solution in order to raise the corresponding H^+ concentration in the vicinity of the positively charged carrier.

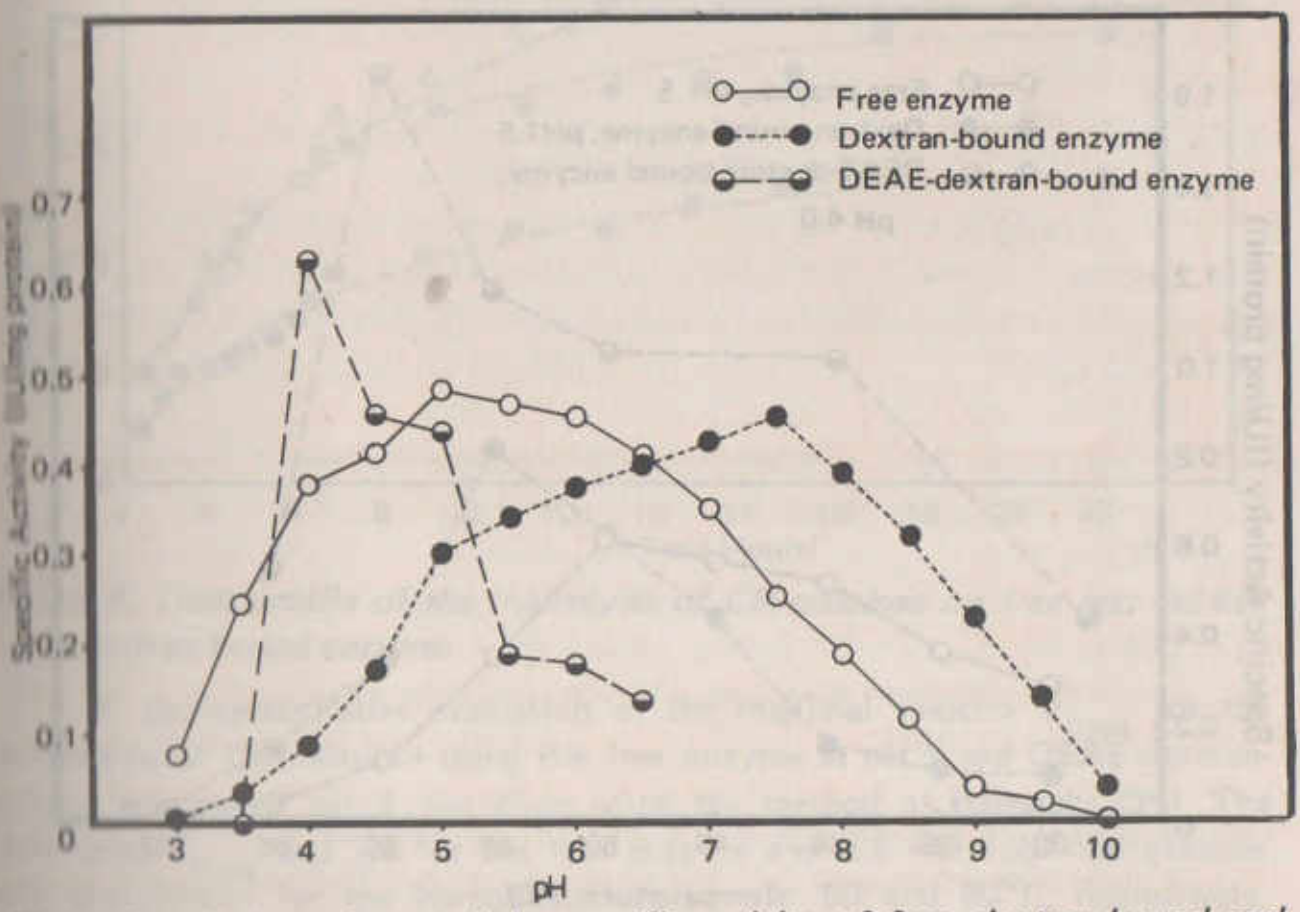

Figure 2. pH dependence of the specific activity of free, dextran-bound and DEAE-dextran-bound carboxymethyl-cellulase at 50°C.

On the other hand, dextran-bound CM-cellulase exhibited a displacement of its pH activity profile towards more alkaline pH values. This pH displacement is commonly observed for enzymes covalently bound to negatively charged carriers. It could be caused by localized electrostatic interactions brought about by chemical reactions employed for immobilization or by an enzymatically generated pH gradient between the domain of the enzyme-carrier conjugate and the external solution (23). A more definitive explanation warrants additional data such as the effect of ionic strength on the pH activity profile. Nevertheless, the pH displacement in the present study has been observed for several enzymes which were covalently attached to neutral carriers (23) and is not an uncommon finding.

The temperature profiles of CM-cellulase before and after binding to the soluble carriers at their optimal pH values are presented in Figure 3. The free enzyme has maximal activity at 50°C while the enzyme bound to dextran and DEAE-dextran has temperature optima of 55 and 60°C, respectively. Stability tests were also conducted on both free and dextran-bound enzymes at pH 5.0 and 7.5, respectively, by incubation at 60°C for 12 hours. The residual activity of the free enzyme was observed to be less than one-tenth that of the immobilized enzyme (24). The enhanced thermal stability of immobilized CM-cellulase may be explained in terms of the effect of the carrier in stabilizing the active conformation of the enzyme via covalent and non-covalent interactions. Similar results of thermal stabilization after enzyme immobilization have been reported in the literature (23).

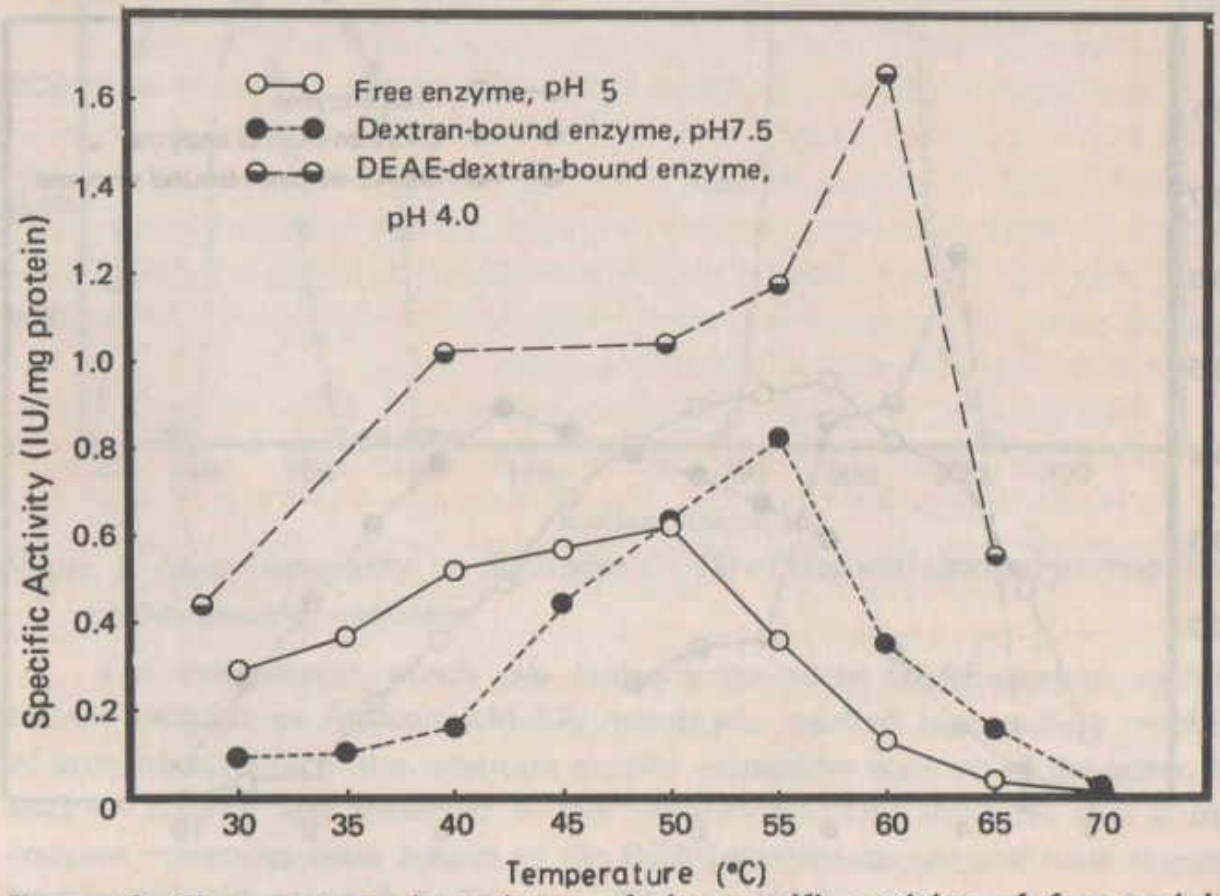

Figure 3. Temperature dependence of the specific activity of free and immobilized CM-cellulase at their optimal pH values.

The time profile of the hydrolytic action on carboxymethyl-cellulose by free enzyme at pH 5.0 and by DEAE-dextran-bound enzyme at pH 4.0 is shown in Figure 4. At 50°C the immobilized enzyme exhibited an initial activity, i.e. initial slope of the curves in the figure, which was 40% less than that of the free enzyme. Similarly the initial activity of the bound enzyme was 37% lower at 50°C relative to the value at 60°C. The latter observation illustrates the effect of increasing temperature in facilitating reactions, as embodied in the rule of thumb that reaction rates increase by a factor of two or three for each 10°C rise in temperature (25). The lower initial activity of the bound enzyme compared to the free enzyme at the same temperature may be attributed to restricted access of the substrate to the immobilized catalyst due to the polymeric nature of both substrate and carrier. A film of essentially stagnant liquid surrounds the latter and only by diffusion through this film barrier can the substrate molecules come in contact and react with the enzyme.

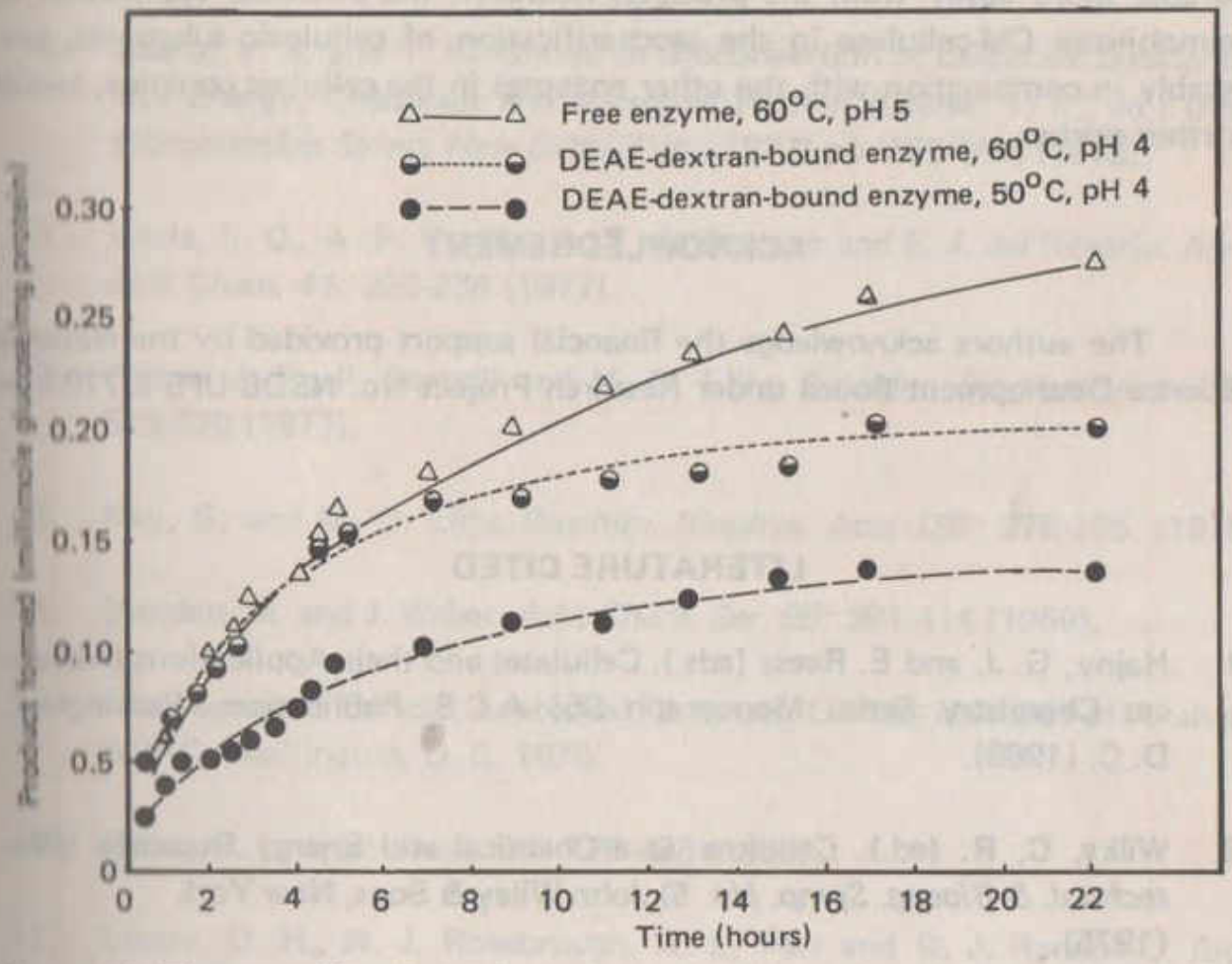

Figure 4. Time profile of the hydrolysis of CM-cellulose by free and DEAE-dextran-bound enzyme.

A semi-quantitative evaluation of the maximal velocity V_{max} for the hydrolysis of CM-cellulose using the free enzyme at pH 5 and DEAE-dextran-bound enzyme at pH 4 was done using the method of Halwachs (26). The estimated V_{max} was 2.3 for the free enzyme and 0.2 and 1.2μ mol glucose/mg protein-min for the immobilized enzyme at 50 and 60°C, respectively. The same numerical values are obtained if the activity is expressed per gram of DEAE-dextran since the loading factor is one mg protein per gram carrier.

These activity values are substantially higher than that reported by Manecke (7) for resin-bound cellulase at 37°C and pH 5.6, namely 0.024 μ mol glucose per minute per gram of immobilized enzyme. For the free enzyme, however, our observed V_{max} value is slightly smaller than the literature value of four μ mol CM-cellulose hydrolyzed per mg protein per minute (19). Values of the apparent Michaelis constant K_m were not estimated in the present study due to the complexities of estimation. The work of Engasser and Horvath (27) shows that the effect of the charged enzymic microenvironment, using a charged carrier, is complex and the notion of an apparent K_m is only applicable if the ionic strength does not change with the substrate concentration.

In summary, immobilization of CM-cellulase on dextran and DEAE-dextran shifts the pH and temperature optima of enzymatic activity. Although the DEAE-dextran-bound enzyme is slightly less active than the free enzyme under optimal conditions, it has greater thermal stability and should be separable more easily from the product. However, the potential application of immobilized CM-cellulase in the saccharification of cellulosic substrates, preferably in combination with the other enzymes in the cellulase complex, awaits further studies.

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