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Carboxypeptidase Immobilization on a Cellulosic Matrix

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ABSTRACT

Immobilization of carboxypeptidase A on periodate oxidized cellulose, as a biocompatible carrier, was investigated. The properties of the enzyme-polymer complex and the release of the enzyme from the polymer were studied. The thermal and chemical stability of the enzyme is enhanced by immobilization, possibly due to stabilization of enzyme conformation.

INTRODUCTION

The concept of enzyme immobilization has found extensive use in biotechnology. While generally the soluble enzyme can be used only in a single operation because it is lost in processing, the immobilized catalyst can be easily recovered and has the obvious advantage of repeated use. Often, immobilization enhances the activity provided it has occurred through the nonactive site of the enzyme. However, deactivation may occur if active sites are involved. The immobilization

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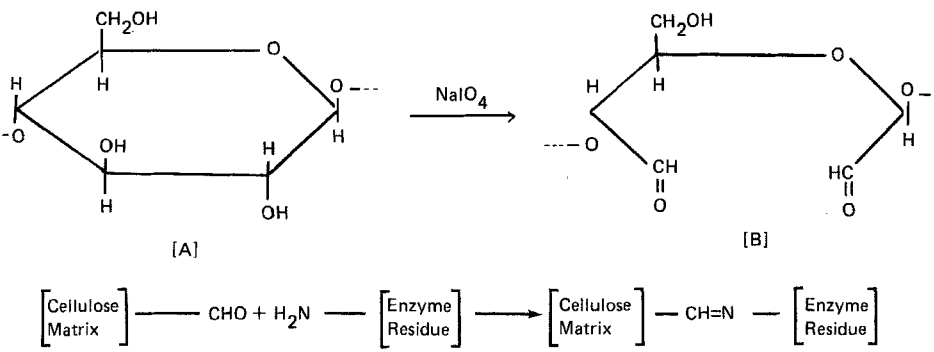
technique has also found application in affinity chromatography. Recently, enzymes immobilized on biodegradable carriers have been tried as drug depots for the slow release of an enzyme in enzyme therapy. The polymer may be either biodegradable or nonbiodegradable and drug may be released by diffusion through the encapsulating polymer matrix [1, 2], by degradation of the polymer matrix, or by a combination of both [3]. The immobilization also protects the enzyme from rapid proteolysis and/or denaturation [4]. Immobilized enzymes have been used to design electrodes for a variety of applications.

The support used for enzyme immobilization must possess groups conveniently linking to the enzyme. The work in our laboratory on oxidized cellulose has indicated that it can form a convenient support whose biodegradability [5] in solutions can be controlled by such factors as degree of oxidation and pH of solution. On oxidizing cellulose with periodate, the glucose moieties in the cellobiose units (A) are converted into dialdehydes (B) [6]. These act as sites for immobilizing an enzyme or any other compound containing amino groups, leading to the formation of a Schiff's base (C) [7]. At high pH, oxidized cellulose hydrolyzes slowly.



Recently, Vasudevan et al. studied the immobilization of α-chymotrypsin on oxidized cellulose and indicated the potential use of this polymer for the preparation of drug delivery systems [8, 9]. In this paper, data on the immobilization and release of carboxypeptidase A are discussed with a view to preparing columns of immobilized carboxypeptidase A for the sequential analysis of peptides. As carboxypeptidases are enzymes that remove amino acids one at a time from the C-terminal of polypeptides and proteins, they are the best tools for C-terminal sequence determination.

MATERIALS AND METHODS

Cellulose powder was from E. Merck, Germany, and NCbz-glycyl-L-phenylalanine was from Sigma, U.S.A. All other chemicals were of BDH analytical reagent grade.

Isolation and Purification of Carboxypeptidase A

Dua and Srivastava purified carboxypeptidase A from goat (the main animal of slaughter in India) pancreas [10] and characterized in this laboratory for its substrate specificity, the effect of ion and ionic strength, buffer effect, and the requirements and role of metal ions [11, 12].

ASSAY FOR CARBOXYPEPTIDASE A ACTIVITY

Colorimetric Method

The carboxypeptidase A activity was determined by the method of Folk and Gladner [14], except that the assay mixture was incubated at 50°C for 10 min. The absorbance of the ninhydrin color product of released amino acid was read at 565 nm against a blank. The specific activity is expressed in enzyme units/mg proteins, one enzyme unit being equal to 1 μM of amino acid liberated per minute under assay conditions.

Preparation of Oxidized Cellulose

Cellulose was oxidized with sodium meta periodata at 25°C in the dark as described earlier [6]. The extent of oxidation was controlled by varying the concentration of periodata (0.1 to 0.4 mol/L) and the duration of reaction (7 to 48 h). The course of oxidation was followed by estimating the oxygen consumption in terms of the decrease of periodate concentration by titration with arsenite [6]. After the required reaction time, the oxidized cellulose was removed from the solution and plunged into a large volume of distilled water. It was washed several times with distilled water to remove the unreacted periodate completely. Samples ranging from 10 to 1000 oxidized units per 100 cellobiose units were prepared (degree of oxidation: 10-100%). The degree of oxidation of the samples used for the present experiments was 50%, unless otherwise stated.

Immobilization of Carboxypeptidase A

About 500 mg of carrier was equilibrated with 6 mL of a solution of carboxypeptidase A (1.42 mg/mL) in acetate buffer of various pH (4-10) for different periods of contact time (2-24 h). The mixture was stirred occasionally and the reaction was carried out at 4°C. The immobilization was found to be maximum at pH 6.0 for a fixed period of time. The product was washed with 0.05 M acetate buffer (pH 6)

and distilled water in succession. The amount of bound enzyme was determined by measuring the absorbance of the supernatant at 278 nm before and after the reaction. The enzyme activity was determined by the colorimetric method. The immobilized sample (20 mg) was taken for assay.

Formation of Reduced Carrier-Enzyme Complex

The imino groups were reduced by taking immobilized carboxypeptidase A (250 mg) with sodium borohydride (200 mg) in 0.2 M phosphate buffer of pH 6.0. The mixture was stirred at 250°C for 2 h. The completion of the reaction was checked by adding 2-3 drops of 0.01 M acetic acid. The reduced sample was washed with distilled water twice. Finally, it was dried and the activity was checked.

Thermal Stability of the Immobilized Enzyme

The immobilized enzyme samples were withdrawn every 15 min and their activity was determined. Similar experiments were repeated with the native enzyme.

Repeated Activity of the Immobilized Enzyme

A sample (20 mg) was taken and the enzyme activity was determined in the same sample 4-5 times after every 24 h.

Determination of the Michaelis-Menten Constant

K_m of immobilized enzyme and free enzyme were determined. Samples (20 mg) of immobilized enzyme were taken in different test tubes, and the activity was determined with different concentrations of substrate.

Determination of the Half-Life of the Enzyme

The immobilized enzyme samples (20 mg) in different test tubes were incubated at 50°C. Activity was checked every 30 min. Similar experiments were repeated with the native enzyme where activity was determined every 5 min.

RESULTS AND DISCUSSION

Conditions of Immobilization

Figure 1 shows the extent of immobilization as a function of time at pH 6.0. Maximum immobilization was achieved at 8 h contact time. The fall in the amount of bound enzyme beyond this period may be partly due to the degradation of the matrix itself, thus releasing some of the enzyme along with it. The effect of pH on the amount of immobilized enzyme is also shown in Fig. 1. It is seen that maximum immobilization occurred around pH 6.0 and was negligible at low pH.

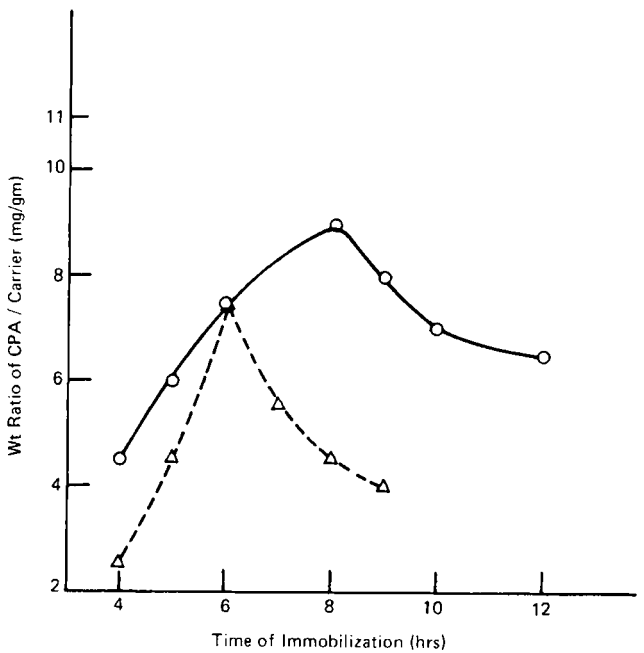


FIG. 1. Amount of immobilized CPA (mg/g) of the carrier vs time (0.05 M acetate buffer, pH 6.0, 6 mL of 1.42 mg/mL solution of enzyme reacted with 500 mg of oxidized cellulose at 4°C: —) and vs pH (time 8 h and other conditions unchanged: --).

Rate of Enzyme Release

The amount of immobilized enzyme was determined by measurements of the absorbance at 278 nm, since Lowry's test could not be conveniently used due to carbohydrate interference. The protein concentration as well as activity was determined before and after immobilization. Generally, about 6.8 mg of the enzyme was bound by 2 g of the carrier at pH 6 in 8 h (20 mg of immobilized samples were taken for determining the activity). The activities in the free enzyme and immobilized enzyme were found to be 84.86 and 66.84 μmol of amino acid liberated/mg protein/min, respectively. The activity of a given batch was determined every 24 h using a fresh sample for each experiment. From Fig. 2 it is clear that after 6 d the enzyme activity becomes constant in both forms of immobilized enzyme. An unreduced form of the immobilized enzyme with 48% activity was retained whereas one with 56% activity was retained in the reduced form although initially it showed less enzyme activity than the other form.

Figure 3 shows the enzyme activity determined in a given sample by repeatedly using it in subsequent runs for both forms of the immobilized enzyme. The activity decreases slowly and becomes constant after 5-6 d.

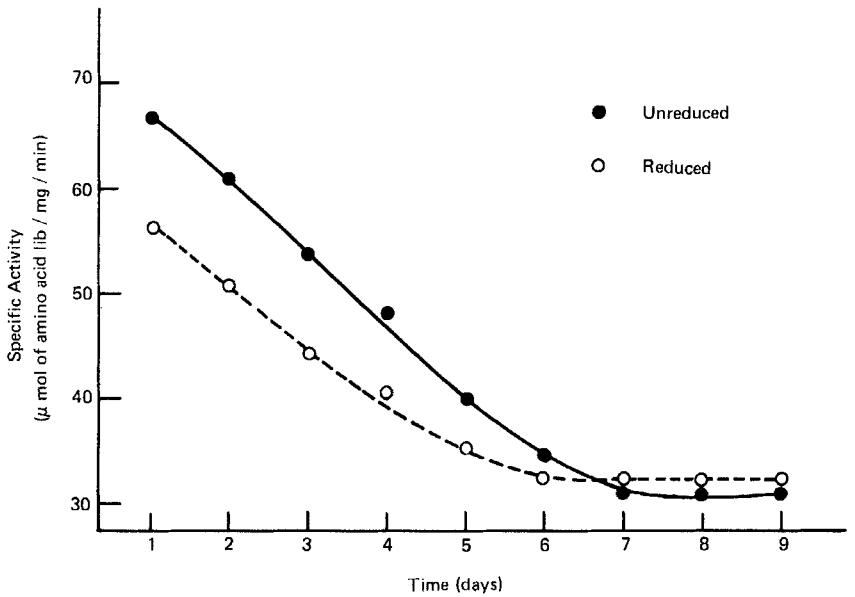


FIGURE 2.

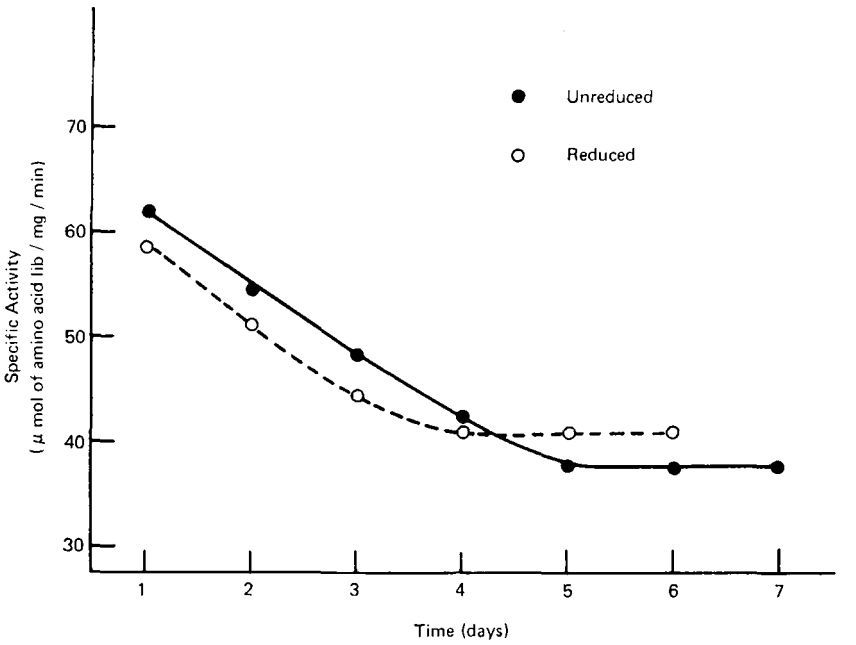


FIG. 3. Repeated activity.

Thermal Stability of the Immobilized Enzyme

As shown in Fig. 4, the activity of the native enzyme was reduced to ~20% of the initial value within 1 h 40 min at 40°C (plot A), at 50°C to ~30% within 30 min (plot B), and at 60°C to ~10% of its initial activity within 25 min (plot C). On the other hand, the activity of the immobilized enzyme was reduced to ~77, 55, and 46% of its initial activity after 3 h at 40, 50, and 60°C, respectively (plots A', B', C').

Thus the immobilized enzyme was found to be less susceptible to thermal deactivation, retaining its activity for a longer time as compared to the native enzyme.

Determination of Michaelis-Menten Constant (K_m) and Half Life

A plot of 1/v versus 1/s for NCbz-glycyl-L-phenylalanine gives a straight line. From Figs. 5 and 6 it is evident that K_m of the free and immobilized enzyme are 0.028 and 0.021 M, respectively.

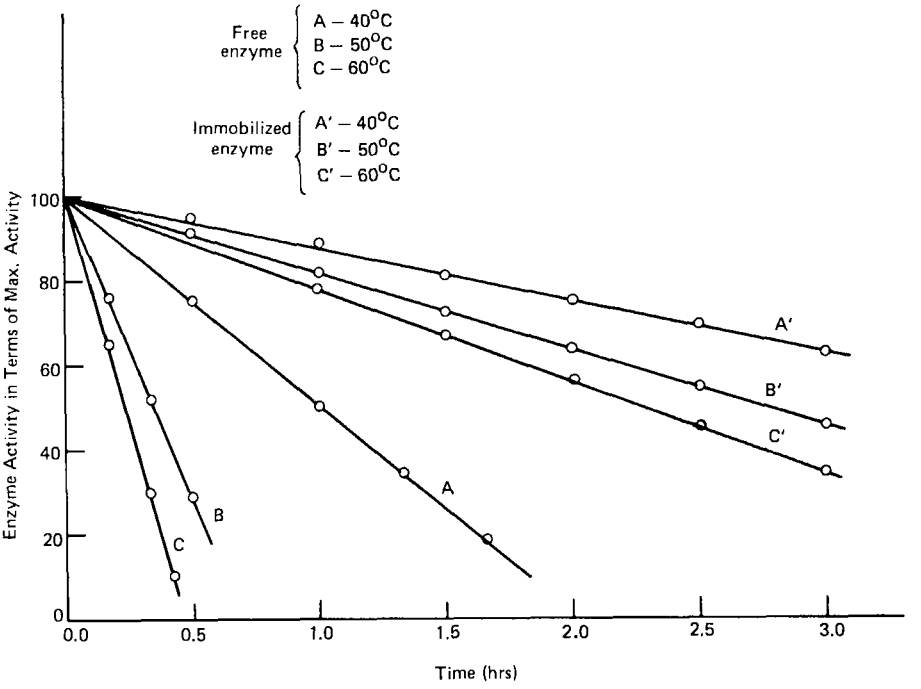


FIG. 4. Thermal stability.

The half-lives of free and immobilized enzyme were determined to be 21 min (Fig. 7) and 165 min (Fig. 8), respectively. This shows that immobilized enzyme is more stable and retains its activity for a longer period.

CONCLUSIONS

The above studies have shown that carboxypeptidase A can be conveniently immobilized on oxidized cellulose. This increases its stability to heat and certain chemical reagents, such as sodium borohydride. Considering that the carrier is biocompatible, as established earlier [5], the pattern of immobilization and release makes the system suitable for the controlled release of enzymes and other immobilizable amino acids for therapy. Preliminary studies (to be published) have indicated the usefulness of immobilized carboxypeptidase in the sequential analysis of peptides.

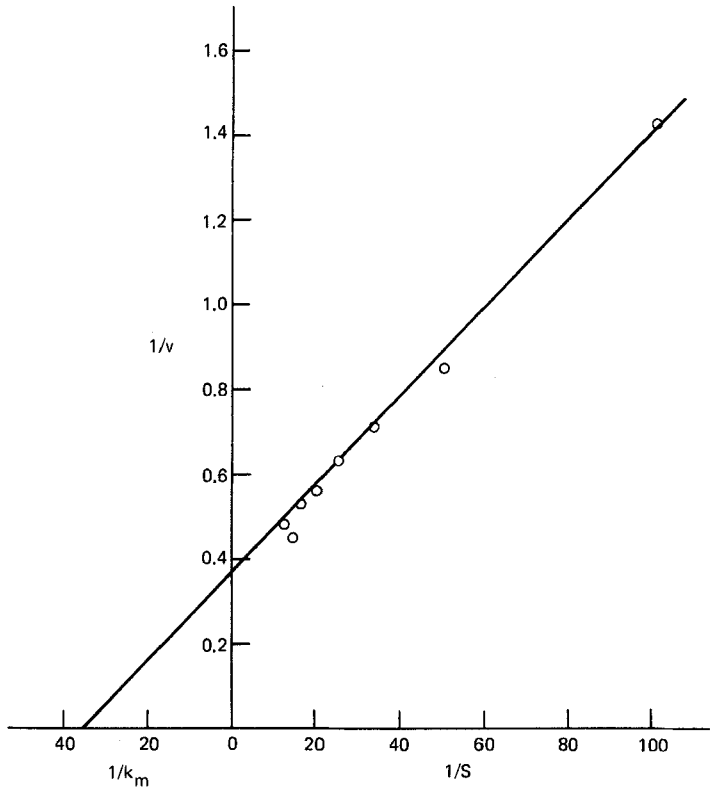


FIG. 5. K_m of free enzyme carboxypeptidase A is 0.028 M.

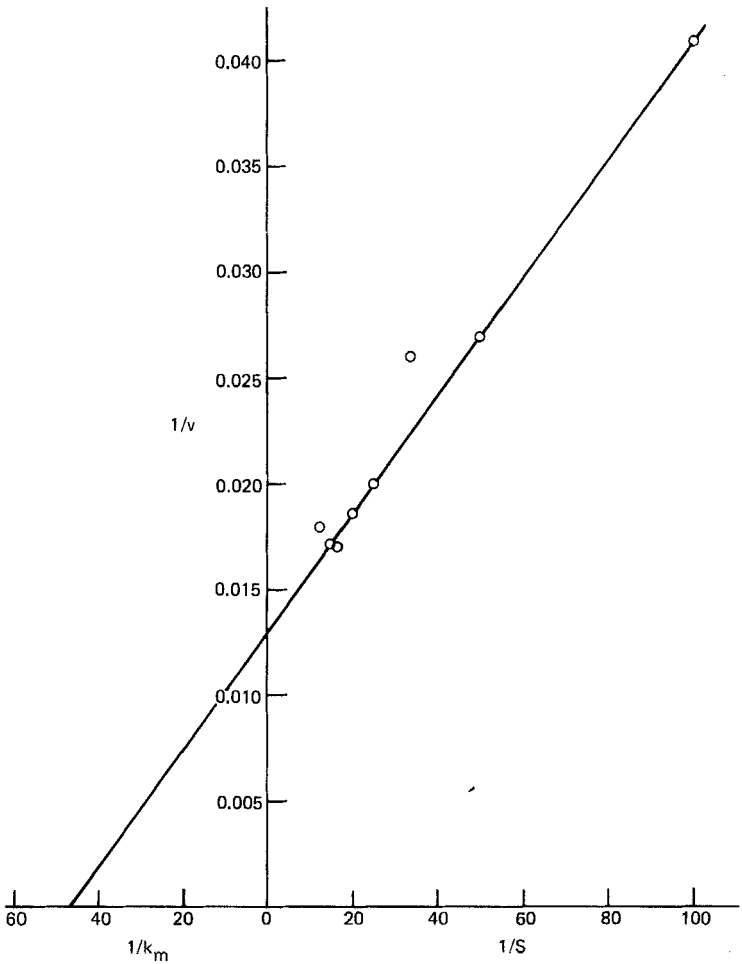


FIG. 6. Enzyme carboxypeptidase immobilized on oxidized cellulose is 0.021 M.

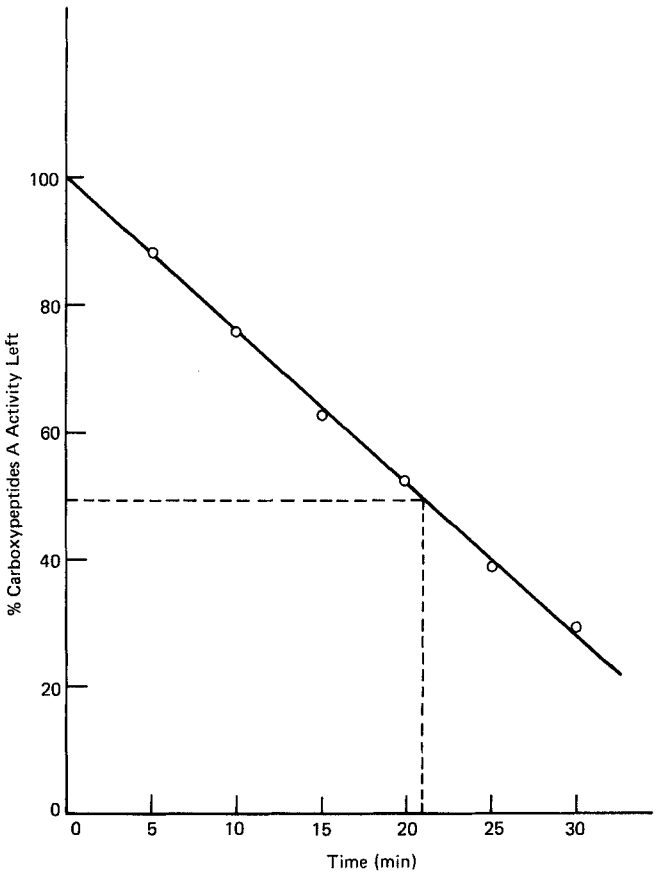


FIG. 7. Half-life of free enzyme carboxypeptidase A at 50°C is 21 min.

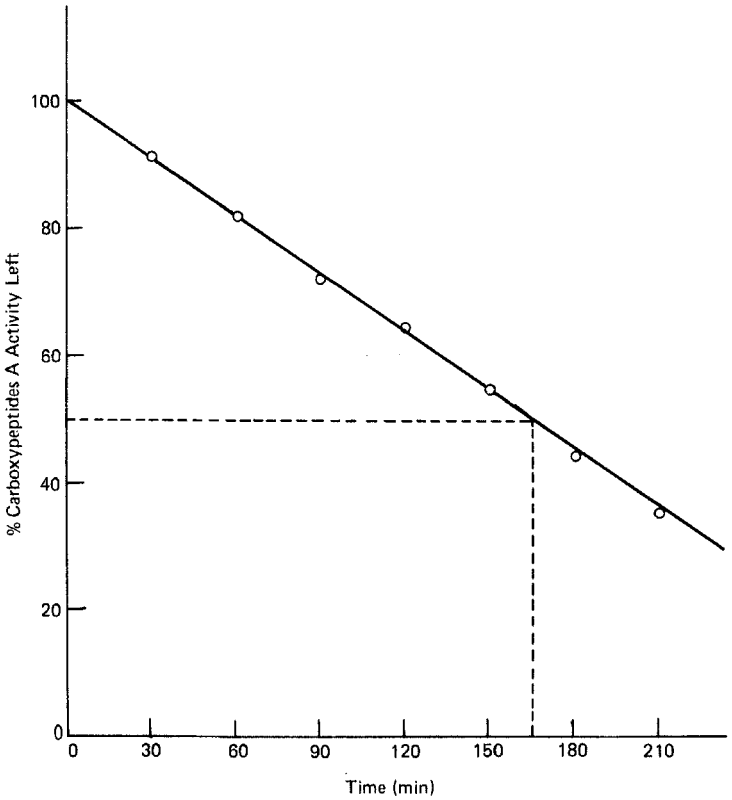


FIG. 8. Half-life of carboxypeptidase A immobilized on oxidized cellulose at 50°C is 165 min.

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