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T. J. Wilkinson[a]; P. S. Mariano[a]; G. I. Glover[a]
[a] Department of Chemistry, Texas A & M University, College Station, Texas

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Affinity Chromatographic Separations of Chemically Modified α-Chymotrypsins from α-Chymotrypsin

T. J. WILKINSON, P. S. MARIANO, and G. I. GLOVER*

DEPARTMENT OF CHEMISTRY
TEXAS A&M UNIVERSITY
COLLEGE STATION, TEXAS 77840

Abstract

Affinity chromatographic conditions have been developed that allow the separation of chemically modified α-chymotrypsins with reduced esterase activities compared to native α-chymotrypsin. This technique should be useful in purifying modified proteins having altered affinities for an affinity absorbent.

Chemical modification studies on enzymes are often complicated by difficulties in interpreting observations of the loss of catalytic activity since the residual activity could be due to a mixture of unmodified enzyme and inactive modified enzyme, or to a partially active modified enzyme, or to several kinds of modified enzymes with differing activities. Affinity chromatography might be useful in this regard since it potentially is an excellent method for separating fully active enzymes from inactive modified enzymes (*1*) and modified active enzymes provided that they all have differing affinities for the substrate analogs used as affinity adsorbents.

We had the opportunity to apply this technique in a novel fashion during our recent studies of α-chymotrypsin and related modified enzymes. α-Chymotrypsin can be reacted with phenacyl bromide to produce a

*To whom correspondence should be addressed.

modified enzyme whose reduced esterase activity results from steric inhibition of the binding of substrates by the covalently attached phenacyl group (*2*). Furthermore, we suggested that irradiation of the modified enzyme leads to a photoaffinity labeled enzyme(s) with partial activity(ies) and to fully active, apparently native, α-chymotrypsin (*3*). We would like to report our recent affinity chromatographic results which confirm our earlier interpretations.

EXPERIMENTAL

Enzymes and Assays

The source and purification of α-chymotrypsin, the esterase assay used, and the method for preparation of the modified proteins have been reported elsewhere (*3*).

Affinity Chromatography

An affinity column containing D-tryptophan methyl ester as the ligand (*4*) was prepared by coupling the amino acid ester to Affi-Gel 10, an *N*-hydroxysuccinamide ester of succinylated aminoalkyl Bio-Gel A (Bio-Rad), as described by Cuatrecasas and Parikh (*5*). The column (5 × 0.6 cm) was poured and washed with the starting buffer indicated in the figure legends. Eluate was monitored at 280 nm using a Pharmacia UV monitor. The following eluents were used: (A) 0.01 *M* Tris·Cl (pH 8), (B) 0.05 *M* Tris·Cl (pH 8), (C) 0.1 *M* Tris·Cl (pH 8), and (D) 0.1 *M* acetic acid (pH 3). Further details are recorded in the figure legends.

RESULTS AND DISCUSSION

Gel-filtered α-chymotrypsin (*6*) can be completely separated from inactive impurities by affinity chromatography on the column described in the Experimental section (Fig. 1). A small amount of inactive material not retained by the column was also obtained on rechromatography of the affinity chromatographed protein. This inactive protein arises from continuing autolysis of α-chymotrypsin. Phenacyl α-chymotrypsin was found to be only weakly adsorbed to this affinity column when the same eluent (B) as used to adsorb the native enzyme is employed. As expected, when a Tris-buffer (eluent A) of lower ionic strength is used to apply modified enzyme, it is adsorbed to the column (Fig. 2). Elution with this

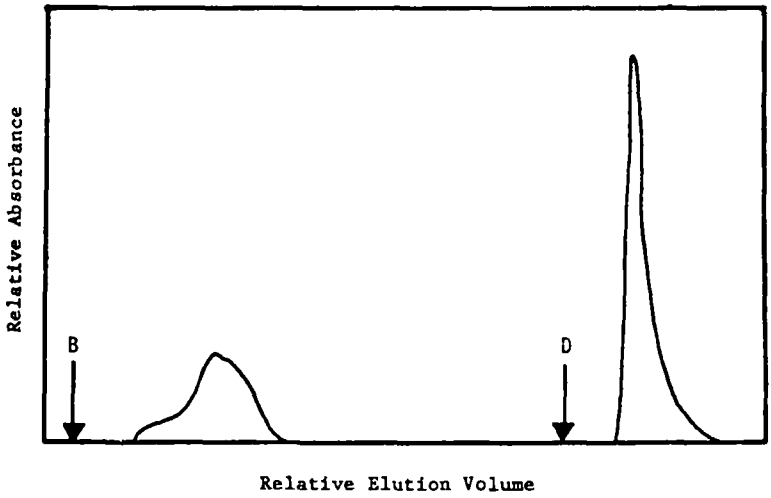

FIG. 1. Affinity chromatography of α-chymotrypsin. α-Chymotrypsin, 0.25 ml of a 5.4 mg/ml solution in eluent B, was placed on the column and eluted with eluent B at a flow rate of 13 ml/hr. After the impurities contained within the first peak were eluted and a stable base line had been reached, the eluent was changed to eluent D (arrow). The first peak was inactive protein while the second peak was α-chymotrypsin.

buffer removed inactive protein present in the α-chymotrypsin used for the modification reaction. A change to eluent C caused elution of a narrow peak containing pure phenacyl α-chymotrypsin. A small quantity of unreacted α-chymotrypsin remained adsorbed until eluent D was used.

On the basis of these results it is clear that the conditions of affinity chromatography can be manipulated in order to cause separation of modified, but still functional, enzyme from native or totally inactive proteins. This feature is further demonstrated by our affinity chromatographic results using irradiated phenacyl α-chymotrypsin (*3*), recorded in Fig. 3. The first peak eluted using eluent A was essentially inactive protein (only 0.2% of the esterase activity of phenacyl α-chymotrypsin). A change to eluent C removed a broad peak with an overall activity 2.2 times that of phenacyl α-chymotrypsin. Evidently this peak is a mixture of more than one modified protein since phenacyl α-chymotrypsin elutes as a sharp peak under these same conditions (Fig. 2). This peak most probably does not contain phenacyl α-chymotrypsin since irradiation had been conducted for periods long enough to insure complete conversion of

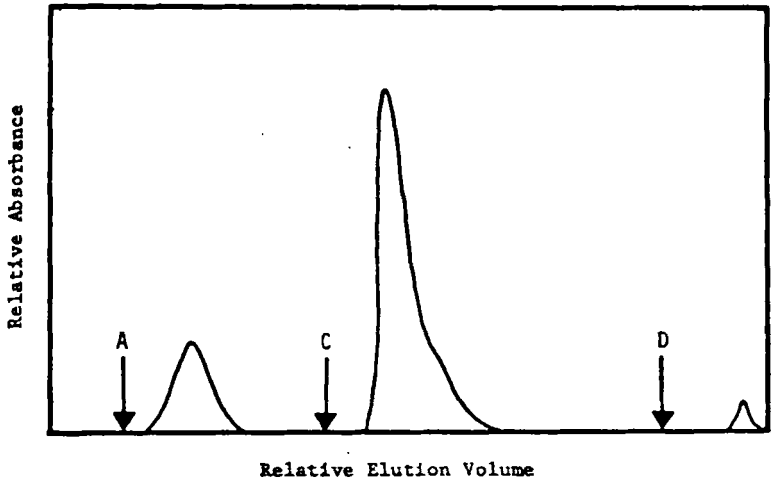

FIG. 2. Affinity chromatography of phenacyl α-chymotrypsin. The modified enzyme, 0.25 ml of a 5 mg/ml solution in eluent A, was placed on the column and eluted with eluent A giving a peak of inactive protein. The eluent was changed to C (arrow) causing the elution of phenacyl α-chymotrypsin. A change to eluent D (arrow) gave a small amount of unmodified α-chymotrypsin.

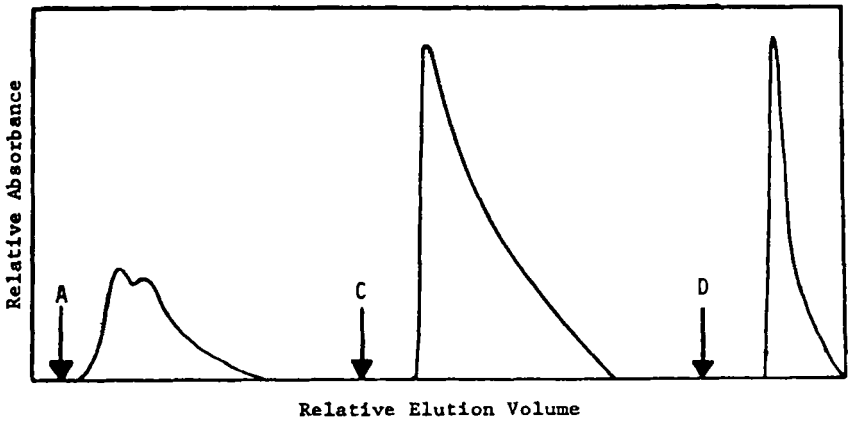

FIG. 3. Affinity chromatography of irradiated phenacyl α-chymotrypsin. Phenacyl α-chymotrypsin, 15 mg in 3 ml of water at pH 3, was irradiated until a maximum in esterase activity was reached. One-half of the solution was adjusted to pH 8 with 0.05 *M* potassium hydroxide and placed on the column. The elution was carried out as in Fig. 2, giving the elution profile shown.

this modified enzyme. Finally, elution at pH 3 with eluent D gave an active protein with the same specific activity as native α-chymotrypsin (4.2 times that of the phenacyl enzyme).

These results are in good agreement with our earlier interpretation that the increased esterase activity noted on photolysis of phenacyl α-chymotrypsin is due primarily to regeneration of α-chymotrypsin by photolytic extrusion of the phenacyl group and that the reason for the incomplete reactivation of the enzyme, evidenced by a low specific activity of the photolyzed enzyme, was due to the formation of a photoaffinity labeled enzyme that still contained the phenacyl group and had a much reduced esterase activity compared to native enzyme.

Cuatrecasas et al. have shown that inactive α-chymotrypsin, affinity labeled with diisopropyl fluorophosphate, could be separated from a small amount of unmodified protein by affinity chromatography. Other applications of affinity chromatography have been reviewed recently (*1*). Our results suggest that conditions for affinity chromatography can be found that will allow the fractionation of proteins with varying affinities for the same substrate which result from different chemical modifications of native enzymes. This type of application is in addition to the use of this method for separation of totally inactive proteins from active enzymes.

Acknowledgments

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