CHEMICAL FIXATION OF ELASTASE TO AGAROSE

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1. Introduction

The preparation of water insoluble enzymes as well as the kinetics of the polymer-bound enzymes have been described [1–4]. Insoluble enzymes have also been used to isolate inhibitors from different sources by specific adsorption [5–10].

This report describes the chemical coupling of elastase to agarose activated by cyanogen bromide, and some properties of the bound enzyme.

2. Material and methods

Porcine pancreatic elastase (Pancreatopeptidase, EC 3.4.4.7, 2 X crystallized in water) and elastin Congo Red were obtained from Seravac (England); Trypsin, Chymotrypsin, *N*-Acetyl-L-tyrosine ethylester (AcTyrOEt), Tosylargine ethylester (TAME) from Sigma (USA); *N*-Acetyl-L-alanyl-L-alanyl-L-alanine methylester ($AcAla_3OMe$) from Sigma (USA); Agarose (Sepharose 2B, 4B, 6B) from Pharmacia (Sweden) and cyanogen bromide was purchased from Fluka (Switzerland).

2.1. *Coupling of elastase to agarose*

Chemical fixation of elastase to agarose was done by the cyanogen bromide method [2, 3]. The general procedure for coupling the enzyme to the different agaroses (Sepharose 2B, 4B, 6B) was as follows:

Sedimented agarose corresponding to approx. 1500 mg dry gel was washed with distilled water on a glass filter and suspended in a solution containing 1 g BrCN per 25 ml distilled water. During the 8 min activation the pH was maintained at 11.5 with 1 M NaOH using a pH-Stat (Radiometer pH meter 51 with Titrator 11 and autoburette unit ABU 12). The activated gel was washed with water and 0.1 M $NaHCO_3$ until free from unreacted BrCN. The enzyme was coupled by suspending the activated gel in a solution of elastase (50 mg elastase/25 ml 0.1 M $NaHCO_3$ buffer, pH 8.5). The reaction mixture was stirred gently for 20 hr at 5°. The coupled product was then washed thoroughly by the method of Axén [4].

2.2. *Determination of amount of bound enzyme*

The amount of fixed enzyme was determined by amino acid analyses according to Spackman, et al. [10]. The molecular weight 25,000 was used for elastase to calculate the degree of substitution. Table 1 shows the coupling yield to different agarose gels.

2.3. *Assay of enzymatic acitivity*

Elastolytic activity was assayed by the Congo-Red elastin method [11, 12].

Esterase activity toward AcTyrOEt, TAME and $AcAla_3OMe$ were estimated titrimetrically [13–15] with a pH-Stat at 23°. The reaction volume was 2 ml and the substrate concentrations were 10 mM, 5 mM and 5 mM, respectively. The electrodes were rinsed with detergent between each determination and washed with 10^{-3} M HCl and distilled water. The slope of the tangent to the initial part of the curve obtained when titrated with 0.02 M NaOH, was taken as a measure of the rate.

Proteolytic activity was determined with casein as substrate according to Bergmayer [16] in 0.1 M carbonate–bicarbonate buffer, pH 8.8. The casein concentration was 1%. One ml substrate, 1 ml buffer and the enzyme were incubated in test tubes at 37°

Table 1
Chemical coupling of elastase to cyanogen bromide-activated agarose.

Polymer	Amount of enzyme in the reaction mixture per 1500 mg dry gel (mg)	Enzyme bound per g dry conjugate (mg)	Coupling yield based on amount of enzyme added (%)
Sepharose 2B	50	12.5	37.5
Sepharose 4B	50	25	80
Sepharose 6B	50	33	99

Table 2
Activity of bound elastase toward casein and $AcAla_3OMe$ substrates.

Polymer	Activity toward $AcAla_3OMe$		Caseinolytic activity	
	pH optimum	Activity ratio bound to free enzyme (%)	pH optimum	Activity ratio bound to free enzyme (%)
Sepharose 2B	8.7	37	9.0	27
Sepharose 4B	8.5	35	9.0	26

Activity of free elastase at 23° toward $AcAla_3OMe$ was 32 μmoles min^{-1} mg^{-1}. The pH optimum in carbonate buffer was pH 7.5.

for 20 min with mild stirring. Three ml 5% trichloroacetic acid was then added to stop the reaction and the samples were allowed to stand for 30 min. The precipitate formed was filtered off and the filtrate centrifuged. The absorption at 280 nm of the supernatant was plotted against the amount of enzyme. The slope of the tangent to the initial part of the curve was again taken as a measure of activity.

To determine the activity of bound enzyme, only casein and $AcAla_3OMe$ could be used. The suspension of coupled enzyme was stirred and samples were withdrawn by pipetting gel into weighed glass filters and washing with distilled water and acetone. The filters were then reweighed to give the weight of the gel by difference.

3. Result

$AcAla_3OMe$ is a highly specific substrate for elastase [15], and is therefore very suitable for determining the activity of insolubilized enzyme when it is not possible to use elastin Congo Red. The enzyme preparation which was used showed a slight activity toward ATEE indicating some contaminating enzyme.

Table 2 shows the activity of coupled elastase compared to the free enzyme with both casein and $AcAla_3OMe$ as substrates. The activity toward $AcAla_3OMe$ was determined at pH 7.5 and towards casein at pH 8.8. The activity of bound elastase toward the high molecular weight substrate shows a lower value than toward the low molecular weight substrate, probably due to different degrees of diffusion into the gels by the two substrates. The apparent pH optima of the conjugates were measured. Fig. 1 shows that there is a shift to an alkaline optimum when the en-

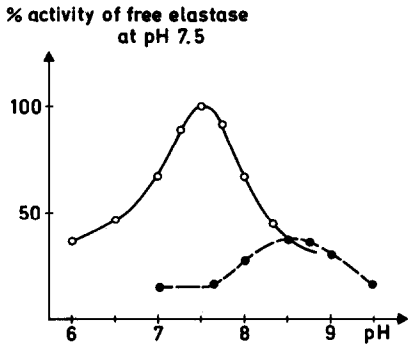

Fig. 1. pH optimum curves for elastase and elastase-agarose. The activity was estimated toward acetyl-L-alanyl–alanyl–alanine methylester (5 mM solution). Amount of fixed enzyme 25 mg/g conjugate. Activity ratio bound: free enzyme 35%. (o–o–o) free elastase; (•–•–•) elastase-agarose (4%).

zyme is coupled, in agreement with results obtained for coupled trypsin, chymotrypsin and papain [4]. The optimum of free elastase is pH 7.7 and is displaced to about pH 8.6 for the coupled enzyme.

3.1. *Stability of free and bound elastase*

Free and bound elastase were incubated at 37° and pH 7.5. Activities were measured on hourly samplings.

Fig. 2 shows that the coupled enzyme was more stable at this temperature and pH than the free enzyme. The loss of activities after 18 hr were 15% for bound and 50% for free elastase.

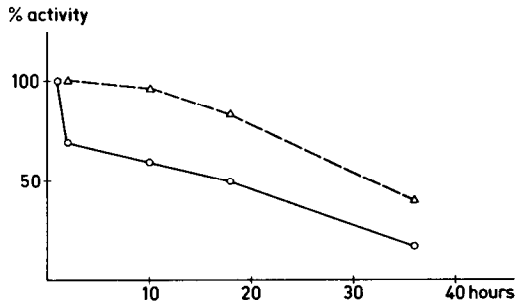

Fig. 2. Stability of free and coupled elastase at 37° and pH 7.5 Free elastase: (o–o–o); bound elastase: (△- -△- -△).

Enzyme conjugates were also stored at room temp and pH 3.0 for 5 days without loss of activity, and at 5° at pH 8.8 for 6 months with 10% loss of activity. The insolubilized elastase has been used to isolate a specific elastase inhibitor in potatoes by specific adsorption. This will be published in a separate paper.

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