# ACTIVE SITE TITRATION OF IMMOBILIZED CHYMOTRYPSIN WITH A FLUOROGENIC REAGENT

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## 1. Introduction

Upon fixation to insoluble matrices, enzymes can change their original properties because of a new microenvironment [1] or because of changed molecular properties [2]. Therefore, the measurement of the activity or the determination of the amount of bound protein do not suffice for completely characterizing the immobilized conjugate; in addition, the amount of functional enzyme in the conjugate should be determined.

For soluble enzymes, a number of reagents have been described which allow the photometric determination of the concentration of active enzyme [3,4]. One of these has been used to measure the amount of functional trypsin in an immobilized trypsin–glass conjugate [5]. Whereas for the assay of soluble enzymes at least 1–2 nmol of enzyme are needed, this figure increases to 10 nmoles for the insoluble enzyme. Also, enzyme particles must be prevented from entering the optical system by use of a special set-up.

Recently, fluorogenic active site titrands for serine proteases have been described which allow the determination of as little as 0.02 nmol of enzyme [6]. In the present communication, one of these compounds has been used to measure spectrofluorometrically in a rapid and reproducible way the amount of active chymotrypsin present in chymotrypsin–Sephadex derivatives.

## 2. Materials

Chymotrypsin–Sephadex was prepared by coupling bovine α-chymotrypsin (NOVO) to Sephadex G-200®, activated with cyanogen bromide at pH 10.3 [7], and was stored as a suspension in water at a concentration of 6 mg conjugate per ml. The conjugate contained 5.4 μmol chymotrypsin per gram as determined by amino acid analysis.

For soluble chymotrypsin, a molar extinction coefficient $\epsilon_{280\,nm} = 4.9 \times 10^4\ cm^{-1}\,M^{-1}$ was assumed

MUTMAC (4-methylumbelliferyl-*p*(*N,N,N*-trimethylammonium)cinnamate) was synthesized according to Jameson et al. [6].

4-Methylumbelliferone was recrystallized from water/ethanol when used as fluorescence standard.

## 3. Methods

Fluorescence measurements were carried out in a Turner Spectro 210 Spectrofluorometer in a 1 cm cuvette. The samples were illuminated at 360 nm, and emission was followed at 450 nm. The combination of these two wavelengths gave the maximal instrument response.

To 2.0 ml of 0.1 M sodium borate buffer pH 7.5 in the fluorescence cuvette, 100 μl of a solution of MUTMAC (0.2 mM in water; final concentration 0.01 mM) was added, and a baseline was recorded. Then, between 50 and 200 μl of the well-stirred chymotrypsin Sephadex suspension was added, and the contents of the cuvette were stirred for 30 sec. The fluorescence due to liberated 4-methylumbelliferone was followed with time, and the plateau value reached after about 1 min (when most of the gel particles had settled to the bottom of the cuvette) was used to calculate the concentration of active sites. Under these conditions, less than 5% of the observed fluorescence is due to

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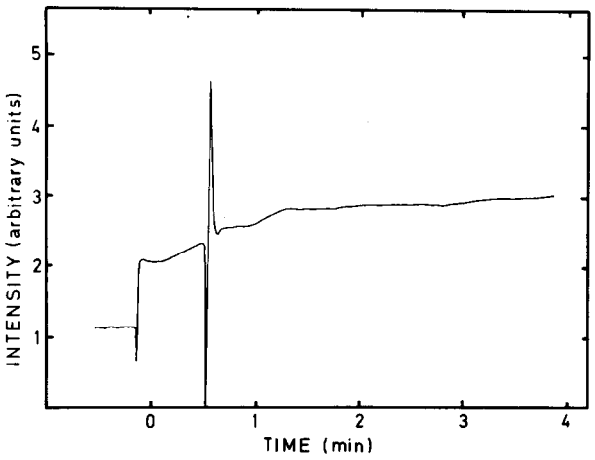


Fig.1. Spectrofluorometric titration of 50 μl chymotrypsin-Sephadex with MUTMAC. The recorder trace previous to t=0 shows the fluorescence intensity of the reagent (0.01 mM MUTMAC in 0.1 M sodium borate buffer pH 7.5; total vol 2.1 ml). At t=0 the enzyme suspension was added to the reagent, and stirred for 30 sec. The recorder trace during that period does not represent a fluorescence signal. Then, the fluorescence due to liberated 4-methylumbelliferone was followed. The excitation wavelength was 360 nm, and the emitted fluorescence was analysed at 450 nm.

enzymatic post-burst hydrolysis. A typical recorder tracing of the fluorescence is shown in fig.1.

The calculated concentration of active enzyme was independent of the amount of suspension or MUTMAC added, and was constant after stirring for 15 sec up to 1 min.

## 4. Results and discussion

With the method described here, a concentration of active chymotrypsin of 2.9 ± 0.2 μmol/g conjugate was determined, as compared to 5.4 μmol/g calculated from amino acid analysis. Thus, the immobilized protein contained 46% inactive material, which must be compared with the value of 21% for the original preparation used for coupling, determined from UV absorption and active site titration.

The reproducibility of the assay was only slightly inferior to that for the soluble enzyme. The sensitivity of the assay, which has been carried out here with about 1 nmol of enzyme, can be increased by a factor of around 100 without reaching the sensitivity limit of the instrument. Then, however, reproducible sampling of the enzyme suspension may be the limiting factor, when conjugates with a high enzyme conten are to be assayed.

The method described here is both simple in its application and requires only small amounts of enzyme It is more sensitive by about one or two orders of magnitude than the spectrophotometric method described by Ford et al. [5] for immobilized trypsin. This is due to the greater inherent sensitivity of fluorometric concentration determinations. Also, scattering of the exciting light by gel particles does not interfere with the signal observed. Fluorogenic active site titrands have been synthesized for a number of serine proteases [6], and can probably be designed for several other classes of enzymes. Therefore, the method described here seems to be applicable not only for chymotrypsin, but for several other enzymes as well. It can thus complement the available tools for the investigation of immobilized enzymes.

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