THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF SEPHAROSE-BOUND L-PHENYLALANINE 4-NITROANILIDE

H.-D. JAKUBKE and L. LANGE

Martin-Luther-Universität Halle-Wittenberg, Sektion Biowissenschaften, Wissenschaftsbereich Biochemie, 402 Halle/S., GDR

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1. Introduction

Enzyme reactions in cells and tissues generally take place in homogeneous aqueous solutions. In contrast to this some enzyme reactions take place in heterogeneous media, e.g. within gels and at interfaces. The utilization of insobilized enzymes [1] and also the use of matrix-bound inhibitors and cofactors in affinity chromatography [2] can serve as models for enzyme reactions in heterogeneous systems.

Observations in our laboratory indicated that an enzymatic cleavage of carrier-bound biologically active substances of low molecular weight is fundamentally possible [3]. As part of a general model study of enzymatic reactions with insoluble substrates we investigated the α-chymotrypsin-catalyzed hydrolysis of Sepharose-bound L-phenylalanine 4-nitroanilide. As spacer, 1 or 2 mol of 6-aminohexanoic acid, respectively, were inserted between the gel matrix and the low-molecular weight substrate.

2. Materials and methods

Sepharose 4B was purchased from Pharmacia, Uppsala, and chymotrypsin from Spofa, Prague.

* *Abbreviations:* Boc-ϵAhx = tert.-butyloxycarbonyl-6-aminohexanoic acid; Phe-Na = L-phenylalanine 4-nitroanilide; ϵAhx-Phe-NA = 6-aminohexanoyl-L-phenylalanine 4-nitroanilide; $(\epsilon Ahx)_2$-Phe-NA = 6-aminohexanoyl-6-aminohexanoyl-L-phenylalanine 4-nitroanilide; Glt-Phe-NA = *N*-glutaryl-L-phenylalanine 4-nitroanilide.

2.1. *Synthesis of the 4-nitroanilides*

ϵAhx-Phe-NA-hydrochloride* (I) was synthesized from Phe-NA and Boc-ϵAhx with a yield of 73% using the mixed anhydride method. The protecting group was removed with 1 N HCl–acetic acid. In the same way $(\epsilon Ahx)_2$-Phe-NA-hydrochloride could be prepared from (I) with a yield of 68%. Phe-NA was obtained using L-phenylalanyl chloride according to Erlanger et al. [4]. Boc-ϵAhx was synthesized as described by Kusch [5]. Glt-Phe-NA could be prepared by the reaction of glutaric anhydride with Phe-NA [4].

2.2. *Preparation of the Sepharose–substrate-conjugates*

Cyanogen bromide activation of Sepharose 4B was performed according to Axen et al. [6]. 0.5 mM ϵAhx-Phe-NA-hydrochloride and $(\epsilon Ahx)_2$-Phe-NA-hydrochloride, respectively, were dissolved in water, liberated from the hydrochloride with a slight excess of triethylamine, and brought again into solution with acetone. The activated Sepharose gel (20 ml) was suspended in this solution stirred at room temperature for 4 hr. The gel was washed thoroughly with the 50-fold volume of: a) water–acetone 1:1 (v/v); b) 1 M NaCl; c) 0.05 M formiate, pH 3.0, 1 M NaCl; d) 0.1 M Tris–HCl, pH 7.8, 1 M NaCl; and e) 0.05 M Tris–HCl, pH 7.8; until no 4-nitroanilide could be detected in the eluate.

The amount of Sepharose-bound 4-nitroanilides was determined by means of hydrolysis with NaOH [7]. The liberated 4-nitroaniline was measured photometrically at 405 nm.

For the content of Sepharose-bound 4-nitro-

anilides we found 1.9 μM/ml settled gel in the case of εAhx-Phe-NA and 5.1 μM/ml settled gel for $(\epsilon Ahx)_2$-Phe-NA.

2.3. *Enzymatic measurements*

The measurements were carried out in a 0.05 M Tris–HCl buffer with 0.05 M $CaCl_2$ at pH 7.8. The individual amounts of substrate gel were prepared from a stirred 'stock suspension'. The concentrations of the stock suspensions of the two different substrates were equal in relation to the amount of matrix-bound 4-nitroanilides. A batch-type procedure was used to determine the enzymatic hydrolysis at 25°C. Using a continuous method, the increase of absorbance was determined at 410 nm (ϵ_{410} = 8800 cm^2 $mmole^{-1}$) [8] in a thermostated cuvette with continuous stirring. As blank, in every case a suspension containing water instead of the enzyme was checked. Using the discontinuous technique an aliquot was withdrawn by the aid of a special syringe, provided with a nylon membrane. 4-Nitroaniline was determined photometrically at 405 nm (ϵ_{405} = 9620 cm^2 $mmole^{-1}$) [9]. The protein concentration in the supernatant was determined from the absorbance at 280 nm and 405 nm as follows:

$$A_{280}^{\text{protein}} = A_{280}^{\text{total}} - \frac{\epsilon_{280}}{\epsilon_{405}} \times A_{405}^{\text{total}}$$

where: ϵ_{280} = molecular absorption coefficient of 4-nitroaniline. To relate the caiculated absorbance of protein at 280 nm to protein concentration $E_{280}^{1\%}$ = 20.0 [10] was used.

3. Results

3.1. *Time dependence of enzymatic hydrolysis*

The time dependence of hydrolysis of both matrix-bound substrates and Glt-Phe-NA was determined using the continuous procedure at 410 nm. The course of hydrolysis was proportional to time during the first 15 min. About 70% of total bound $(\epsilon Ahx)_2$-Phe-NA was hydrolyzed after 4 hr.

3.2. *Dependence of enzymatic hydrolysis on the amount of Sepharose-substrate gel*

Similar to the behaviour of soluble substrates we tried to study the dependence of the hydrolysis rate on the amount of matrix-bound substrates. For this purpose the discontinuous technique was used (fig. 1). A plot of these data according Lineweaver-Burk gives a straight line.

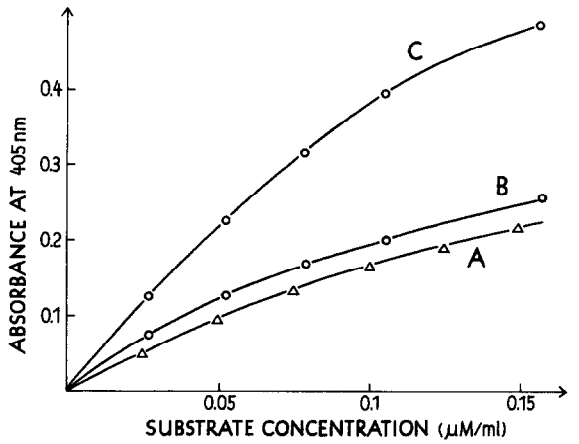

Fig. 1. Substrate-dependence of enzymatic hydrolysis at 25°C: A) Glt-Phe-NA; B) Sepharose-bound εAhx-Ph-Na; C) Sepharose-bound $(\epsilon Ahx)_2$-Phe-NA. Enzyme concentration 100 μg/ml. Incubation time 15 min.

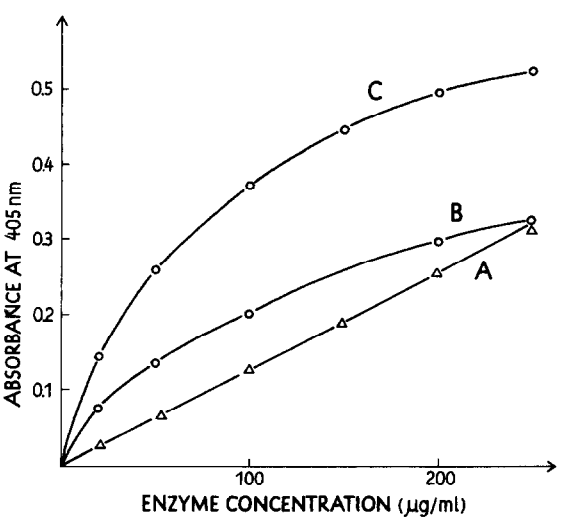

Fig. 2. Dependence of hydrolysis on the enzyme concentration at 25°C. A) Glt-Phe-Na, 10^{-4} M; B) Sepharose-bound Ahx-Phe-NA, 0.1 μM/ml; C) Sepharose-bound $(\epsilon Ahx)_2$-Phe-NA, 0.1 μM/ml. Incubation time 15 min.

3.3. *Dependence of enzymatic hydrolysis on the enzyme concentration*

Using the discontinuous technique the dependence of the hydrolysis rate on the enzyme concentration was studied (fig. 2).

3.4. *Distribution of the enzyme between the gel phase and the buffer phase*

Supporting the assumption that the enzyme concentration in the gel phase is different from that in the buffer phase we determined the decrease of the enzyme concentration in the buffer phase (fig. 3). In this connection the volume of the gel phase is defined, approximately as the volume of the settled Sepharose gel in the reaction suspension. The relation of the enzyme distribution depends both on the amounts of the gel and on the total enzyme concentration. From the experimental data in fig. 3 a protein concentration of 2340 μg/ml gel was calculated for the equilibrium-state. In control experiments with the same amount of untreated Sepharose 4B no increase in protein concentration in the gel phase could be observed.

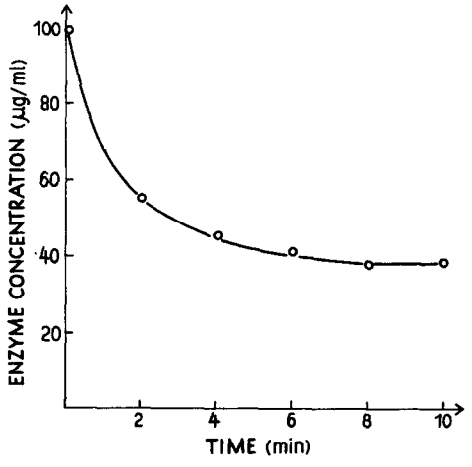

Fig. 3. Determination of the enzyme concentration in the buffer phase at 25°C. Initial enzyme concentration 100 μg/ml. 0.54 μM (εAhx)$_2$-Phe-NA bound to 0.11 ml Sepharose gel/4 ml reaction suspension.

4. Discussion

We started from the assumption that an enzymatic action with Sepharose-bound substrates should occur according to the following scheme:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

In agreement with this the substrate dependence of the hydrolysis rate shows the same course as observed with Glt-Phe-NA. In contrast to the soluble substrate we could not find a straight-line-plot for the dependence of the hydrolysis rate on the enzyme concentration. It seems that during the action of an enzyme molecule with a matrix-bound substrate molecule the surrounding substrate molecules are sterically hindered to react with other enzyme molecules. An increasing enzyme concentration leads to saturation of the gel matrix.

Spacers of various lengths were used, but an investigation with directly bound Phe-NA was not successful.

It is interesting that Sepharose-bound substrates are better hydrolyzed by chymotrypsin than soluble ones. The results described could be interpreted as follows:

1. Using an equal amount of substrate per volume unit, in the case of Sepharose-bound substrates a substrate concentration at the region of the enzyme action results which is higher than in the homogeneous phase.
2. In addition, the reported affinity effect [11,12] of substrate gels induced an enzyme enrichment within the gel phase.

In order to support the latter fact we found a 60-fold enrichment of the enzyme in the gel phase.

Perhaps, these results could be important for model considerations of heterogeneous enzyme reactions in vivo. The effect of the biospecific adsorption of enzymes used for affinity chromatographic purification is in this case connected with biotransformation.

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