RESOLUTION OF RACEMIC ACYL-AMINO ACIDS- ^{14}C BY WATER-INSOLUBLE HOG KIDNEY ACYLASE .

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SUMMARY

For the purpose of continous resolution of racemic N-acyl-amino acids-^{14}C conditions for the preparation and utilization of water-insoluble hog kidney acylase (E.C. 3.5.1.14) were investigated. A stable, highly active water-insoluble acylase was easily prepared by linking the enzyme to DEAE-Sephadex A-50 in Tris-HCl-buffer, μ 0.045; at pH 7.1.

INTRODUCTION

The preparation of L-amino acids with a high degree of optical purity has been successfully achieved by the action of stereo-specific enzymes on racemic amino acid derivatives. For this purpose, N-acyl-amino acids or amino acid amides are mainly used as substrates, which carboxypeptidases or aminopeptidases respectively are capable of acting upon. Among the enzymes used is kidney acylase, first employed by GREENSTEIN *(1)*.

Enzymatic techniques have also been applied to the preparation of labelled amino acids *(2-5)*.

To make enzymic reactions more convenient the use of water-insoluble enzymes has been introduced. TOSA et al. *(6-13)* succeeded in linking aminoacylase from Aspergillus oryzae to basic derivatives of cellulose and Sephadex. Thus, the continuous optical resolution of acyl-DL-amino acids became possible by employing a column packed with insolubilized enzyme. Recently the application of renal acylase bound to DEAE- cellulose was described by BARTH and MAŠKOVÁ *(14)*.

In order to prepare continuously aliphatic L-amino acids-^{14}C we have investigated the optimum conditions for the binding of kidney acylase to DEAE-Sephadex as well as the utilization of this insolubilized enzyme for the optical resolution of acyl-amino acids-^{14}C.

DISCUSSION

To determine suitable conditions for the binding of the enzyme to a matrix the stability of our acylase preparation in aqueous solution was investigated. As shown in Fig.1, the known lability *(15)* was observed, except that the enzyme appeared to be stable in Tris buffer, pH 7,1, for at least 24 hours. Therefore, the coupling should be carried ou in Tris buffer.

By employing Tris buffer, it was possible to obtain a water-insoluble acylase retaining much of the activity of the native enzyme.

On the contrary, preparations in phosphate buffer or under conditions according to the procedure of TOSA et al. for mold acylase resulted in low active derivatives or were much less reproducible.

In comparison with DEAE-Sephadex A-25 DEAE-Sephadex A-50 showed an almost ten times higher protein binding capacity of 65 mg/g for our acylase preparation. The protein concentration (extinction at 280 nm) did no correlate with the enzyme concentration (acylase activity).

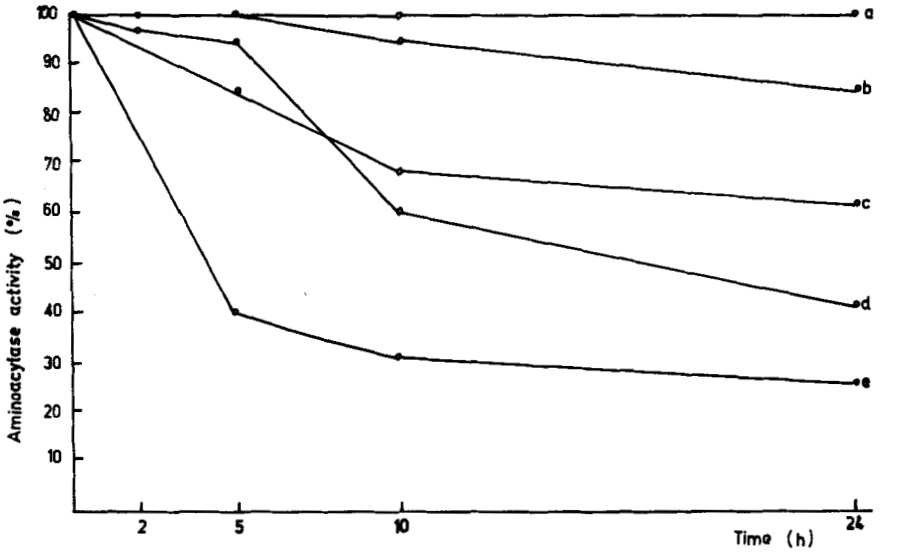

Fig. 1 - Stability of acylase in aqueous solutions

a - Tris buffer, μ 0.045 ; pH 7.1

b - KCl-solution, 0.045M

c - phosphate buffer, μ 0.04, pH 7.1

d - dist. water

e - NaCl solution, 0.9 %

Enzymatically inactive protein remained unbound in Tris buffer, μ 0.045. Such an inhomogeneity had been already shown by MITZ and SCHLUETER *(16)*, who used DEAE-cellulose for enzyme isolation.

More detailed information has been attained with the aid of gradient chromatography of acylase on DEAE-Sephadex A-50 (fig. 2). From fig.2 it becomes obvious that the enzyme remains matrix-bound up to a chloride concentration of about 0,1 molar. When washed with Tris buffer,

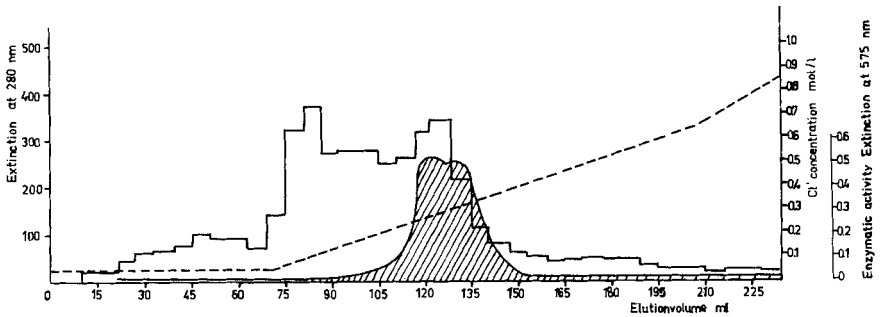

FIG. 2 - Gradient chromatography of hog kidney acylase on DEAE-Sephadex A-50, Tris-Hcl buffer at pH 7.1. (column : 20 x 180 mm, chloride gradient).

potassium chloride solution (0.045 M) or 0.08 M solutions of acylamino acids, no leakage has been observed.

The conversion of acylase into water-insoluble form by linking to DEAE-Sephadex at 4°C did not considerably decrease the enzymic activity. Stability experiments showed that the insoluble acylase was stable for at least a forthnight at room temperature both in Tris buffer and in solution of potassium chloride. With respect to the following chromatographic separation the racemic resolution was carried out in KCl-solution.

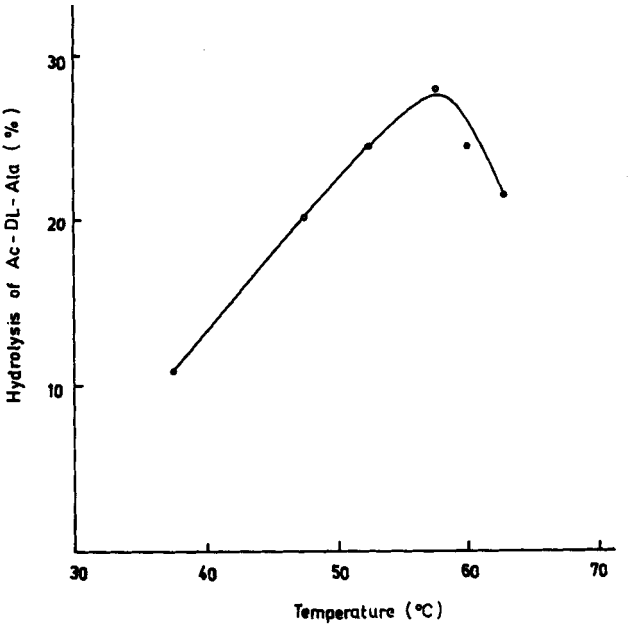

Fig. 3 - Effect of temperature on the hydrolysis of acetyl-DL-alanine by DEAE-Sephadex-acylase column (20 x 180 mm, 38 mg acylase, flow rate : 220 ml/h).

As illustrated in Fig. 3, the hydrolysis rate of acetyl alanine increased up to 100-150 per cent when temperature was raised from 37°C to 57°C. temperature above 60°C caused irreversible decrease of enzymic activity (Fig. 3).

In order to estimate the effect of flow rate of substrate solution on the yield of L-amino acid we assumed that the MICHAELIS-MENTEN-equation can be applied and that neither the size of column nor the kind of distribution of the enzyme in the column affect the kinetic properties.

Thus, we used the following equation derived from the integrated MICHAELIS-MENTEN relation :

$$v_s = \frac{V_{max} \cdot 60}{[P] - 2.3 \log K_m \left(1 - \frac{[P]}{[S_o]}\right)}$$

v_s = flow rate [ml/h]

V_{max} = maximum velocity [µmoles/min]

[P] = concentration of L-amino acid in the effluent

$[S_o]$ = initial substrate concentration

K_m = Michaelis constant [µmoles/ml]

As kinetic data of hog kidney acylase fixed to DEAE-Sephadex are not yet available, those of unbound acylase *(15)* were used. The results of such a calculation and of the experimental measurements are summarized in Table 1.

TABLE 1.

Dependence of the yield of L-alanine as a function of the flow rate

(column 20 x 180 mm) with 38 mg acylase, initial substrate concentration : 0.08 molar

FLOW RATE (ml/h)	YIELD (%) relatively to the L-component	
	calc.	exp.
15	100	100
30	100	100
186	85	87
150	64	51
180	54	39

The example of acetyl-alanine hydrolysis shows a satisfactory result in the range of high yields. The method used gives only crude information, because the kinetic data alter due to the binding of the enzyme, as was shown in the case of mold acylase *(8, 10)*

EXPERIMENTAL

- *Acylase*, lyophilized, was prepared from hog kidneys according to BIRNBAUM et al. *(17)* with an activity of 6500 ± 200 µmoles leucine/mg N-protein, h(substrate : chloroacetyl-leucine, 37°C, amino acid assay according to STEIN and MOORE *(18)*).

- *Preparation of water-insoluble acylase*

  A suspension of 3 g DEAE-Sephadex A-50 in about 200 ml Tris buffer µ 0.045, pH 7.1, was boiled in a water-bath. The gel was then washed with buffer and cooled to 4°C. To the suspension 75 mg acylase was added. The mixture was allowed to stand for half an hour. After removing the supernatant the gel was washed with Tris buffer and then with KCl-solution (0.045 M). The supernatant and washing solutions were checked for absence of enzymic activity.

- *Resolution of N-acetyl-amino acids-^{14}C.*

  25 ml of water-insoluble acylase (≅ 10 mg enzyme) was poured into a column kept at 37°C. The solution of 0.08 M substrate adjusted to pH 7.1 - 7.2 with 0.1 N NaOH was passed through the column at a flow rate as estimated on page 477. For instance, in the case of N-acetyl-alanine-^{14}C a flow rate of 13.5 ml/h and a column with 10 mg acylase were used. After the substrate has passed, the column was washed with KCl-solution. The L-amino acid, and the N-acetyl-amino acid in the effluent of the enzyme column were separated by ion exchange *(19)*. The effluent was passed through a cation exchange resin (KPS Wolfen), which adsobs the amino acid. The elution of the L-amino acid from the resin was performed with ammonia.

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