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PRODUCTION OF L-LYSINE BY IMMOBILIZED TRYPSIN

STUDY OF DL-LYSINE METHYL ESTER RESOLUTION

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Summary

The possibility of producing L-lysine from chemically synthesized DL-lysine has been investigated. Optical resolution of racemic DL-lysine may be achieved by using the stereospecific esterasic activity of trypsin on DL-lysine methyl ester, which gives L-lysine and unchanged D-lysine methyl ester. DL-lysine methyl ester spontaneous hydrolysis may be neglected when operating at pH 5.5 and 30°C. Effect of pH and substrate concentration on hydrolysis rate has been investigated when using as a catalyst either soluble or immobilized trypsin. For this purpose, trypsin was coupled onto an amine porous silica, Spherosil, activated with glutaraldehyde. The optimal pH is 5.8 for soluble trypsin and 6.0 for immobilized trypsin. It was yet possible to lower the apparent optimal pH of immobilized trypsin, and thus increase its activity at pH 5.5, by co-grafting onto Spherosil an aminosilane, for enzyme coupling via glutaraldehyde activation and a positively charged diethyl amino ethyl (DEAE) silane, for decreasing the pH of trypsin microenvironment.

Introduction

L-amino acids, and particularly essential L-amino acids, are important substances in the medical and nutritional fields. Industrial production of amino acids is achieved either by fermentative [1] or by chemically synthetic [2] methods. However, microorganisms stereospecifically produce L-amino acids, when chemical synthesis yields DL-amino acids. In this case, optical resolution of the racemic form is thus necessary for L-form production. Enzymic methods, which have been widely used for optical resolution of racemic amino acids [3,4] do not apply well to L-lysine production, and a physico-chemical method therefore seemed more advisable [5].

We report here on the possibility of applying the stereospecific esterasic

activity of trypsin (EC 3.4.4.4) to optical resolution of DL-lysine methyl ester (DL-LysOMe), in view to convert DL-lysine into L-lysine. Chemically synthesized DL-LysOMe is hydrolyzed by trypsin to give L-lysine and D-LysOMe. L-lysine is precipitated from reaction mixture by methanol addition and D-LysOMe is racemized by heating in acidic conditions and reused for the resolution procedure.

For continuous optical resolution of DL-LysOMe, trypsin has been immobilized onto an amine porous silica, Spherosil, activated with glutaraldehyde.

Materials and Methods

Enzyme. Trypsin from hog pancreas (Sigma Chem. Co., Type IX) was used without any previous purification.

Substrate. DL-LysOMe was chemically synthesized by acid esterification of DL-lysine (Sigma Chem. Co.) with methanol. 300 ml dry methanol were added to 20 g DL-lysine · HCl. Gaseous HCl was bubbled in the thoroughly agitated mixture. The esterification reaction was allowed to proceed for about 3 h. Methanol and HCl were then eliminated under vacuum. Dry DL-LysOMe was stored in a refrigerator. Physico-chemical analysis of the product was performed by NMR and infrared spectroscopy and thin-layer chromatography on cellulose plates (Merck) with a butanol/acetic acid/water (60 : 15 : 25, v/v) solvent. These analyses showed that the esterification yield was equal to 100%, and the product was obtained as a dichlorhydrate. R_F values were 0.17 for DL-lysine · HCl and 0.25 for DL-LysOMe · 2HCl. No cyclic product was detected in the reaction mixture.

DL-LysOMe was prepared from DL-LysOMe hydrolysed with trypsin. 1 g immobilized trypsin (see preparation below) was added to 100 ml of a 2 M DL-LysOMe solution in H_2O at 30°C, pH 5.5. L-lysine produced by trypsin hydrolysis was continuously neutralized by 1 M NaOH, and pH was kept constant. When reaction was complete, immobilized trypsin was allowed to settle down. The supernatant was recovered and 500 ml methanol were added. The mixture was kept at 4°C for 10 h under gentle stirring. L-lysine precipitate was eliminated by filtration and the solution was concentrated to about 100 ml volume, and methanol precipitation was repeated twice. D-LysOMe was then dried under vacuum and washed with methanol.

Trypsin immobilization. Trypsin was immobilized onto a porous silica, Spherosil (Rhone Poulenc Ind.), available as spherical beads (particle diameter ranging from 100 to 200 μm; specific area: 37 $m^2 \cdot g^{-1}$; mean pore size: 800 Å). Spherosil was aminated via γ-aminopropyltrimethoxysilane grafting. Amino-Spherosil and Spherosil co-grafted with aminosilane and diethylaminoethyl-silane (DEAE-silane) were a gift from Rhône-Poulenc (Croix de Berny).

Support activation was achieved by roller-mixing (Denley mixer A 257) 100 mg amino-Spherosil with 20 ml of a 2% glutaraldehyde solution in 0.05 M pyrophosphate buffer (pH 8.6) for 2 h at 25°C. Excess glutaraldehyde was washed off with the same pyrophosphate buffer. Activated support was put in contact with 20 ml of a 2 $g \cdot l^{-1}$ trypsin solution in 0.05 M pyrophosphate buffer (pH 8.6) for 2 h at 4°C. The support was then washed at 4°C four times

with 20 ml of the same pyrophosphate buffer for 30 min, once with 20 ml of a 1 M NaCl in 10^{-3} M HCl for 15 h and 4 times with 20 ml of a 10^{-3} M HCl solution for 30 min.

The amount of immobilized trypsin was determined by measuring the protein content of trypsin solution, before and after immobilization, and of successive washes, by the method of Lowry.

Trypsin activity assay. DL-LysOMe (unless otherwise stated) was used as substrate. L-lysine resulting from trypsin action was continuously neutralized by 0.1 M NaOH addition with an automatic pH-stat device Tacussel TAT4. The pH set point was pH 5.5 and temperature was 30°C. Nitrogen was bubbled through the assay mixture to avoid any carbon dioxide interference. The assay mixture contained 1 ml of trypsin solution in 10^{-3} M HCl (or a suspension of immobilized trypsin in 1 ml 10^{-3} M HCl) and 9 ml of DL-LysOMe solution in water. DL-LysOMe concentration was 0.6 M for soluble trypsin assay and 1.5 M for immobilized trypsin assay. For each assay, a blank without enzyme was measured and, if not negligible, deducted from trypsin assay.

Results

1. Spontaneous hydrolysis of DL-LysOMe

DL-Lys-OMe may undergo a spontaneous chemical hydrolysis, which is favoured by the presence of the ϵ-amino group in the molecule. The spontaneous hydrolysis rate was logarithmically increased with pH, and directly related to ester concentration. At pH 5.5, DL-LysOMe hydrolysis was quite low: the pseudo-first order rate constant was then equal to $0.54 \cdot 10^{-6}$ min^{-1}. All the subsequent experiments have thus been achieved at pH 5.5.

2. Enzymic hydrolysis of DL-LysOMe

Hydrolysis of DL-LysOMe has been catalysed either by soluble or by immobilized trypsin.

Effect of pH. The optimal pH for DL-LysOMe hydrolysis is pH 5.8 for soluble enzyme and pH 6.0 for trypsin immobilized onto glutaraldehyde activated amine-Spherosil (Fig. 1).

Effect of substrate concentration. We have investigated the effect of DL-LysOMe and L-LysOMe concentration on hydrolysis reaction rate. Soluble trypsin was used and its concentration in the assay mixture was $0.2 \text{ g} \cdot \text{l}^{-1}$. From the Eadie-Scatchard plot for L-LysOMe hydrolysis (Fig. 2), the kinetic parameters of trypsin have been determined: K_m = 8.25 mM and V = 14.4 mM · min^{-1}, i.e. k_{cat} = 1680 min^{-1} assuming a trypsin molecular weight of 23 280 [6]. Fig. 2 shows that when DL-LysOMe is used as substrate, the Eadie-Scatchard plot gives a straight line, the intercept of which with the v/[S] axis is the same as for the L-LysOMe curve. On the other hand, the slope and the intercept with the v axis are modified. This is not a consequence of an uncompetitive inhibition of L-LysOMe hydrolysis by D-LysOMe, as the above linear plots could suggest. In fact, D-LysOMe is a competitive inhibitor of trypsin [7]. Furthermore, in the present case, the inhibitor concentration is not constant over the substrate range, but varies with it: initial D-LysOMe concentration is always equal to initial L-LysOMe concentration when using DL-LysOMe as substrate. The Henri-Michaelis-Menten equation for competitive

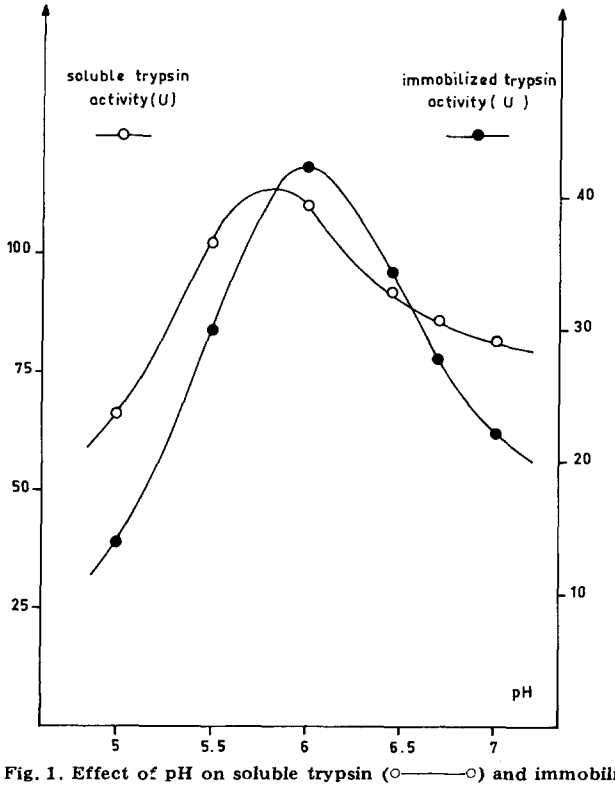

Fig. 1. Effect of pH on soluble trypsin (○———○) and immobilized trypsin (●———●) activity. Soluble trypsin: enzyme concentration was 0.2 g · l^{-1} and substrate concentration was 0.6 M. Trypsin was immobilized onto glutaraldehyde activated amino-Spherosil; 100 mg of trypsin-Spherosil derivative were used and substrate concentration was 1.5 M. Activity was assayed with DL-LysOMe as substrate at 30°C. 1 activity unit (U) corresponds to the liberation of 1 μmol of L-lysine per min in assay conditions.

inhibition may be arranged as follows:

$$V = \frac{v}{[S]}\left\{K_m\left(1 + \frac{[I]}{K_i}\right) + [S]\right\}$$

Assuming [I] = [S], we obtain:

$$V = \frac{v}{[S]}K_m + v\frac{K_m}{K_i} + v$$

Thus:

$$\frac{v}{[S]} = -\left(\frac{1}{K_m} + \frac{1}{K_i}\right)v + \frac{V}{K_m}$$

When compared to the basic velocity equation:

$$\frac{v}{[S]} = -\frac{1}{K_m}v + \frac{V}{K_m}$$

it can be seen that the intercept with the $v/[S]$ axis remains unchanged and equal to V/K_m, while the intercept with v axis is then equal to $V \cdot K_i/(K_m +$

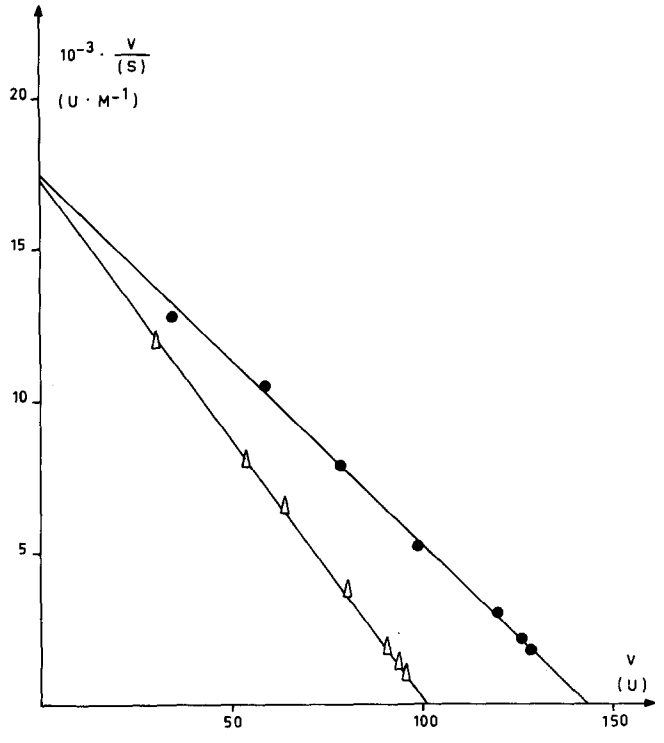

Fig. 2. Effect of substrate concentration on soluble trypsin activity. Trypsin activity was determined with L-LysOMe (●———●) and DL-LysOME (△———△) as substrates, at 30°C and pH 5.5. Enzyme concentration was 0.2 g · l^{-1} and substrate concentration ranged from 0.005 M to 0.4 M. 1 activity unit (U) corresponds to the liberation of 1 μmol of L-lysine per min in assay conditions.

K_i) instead of V. The slope is equal to $-(1/K_m + 1/K_i)$ instead of $-1/K_m$. The inhibition constant for D-LysOMe may thus be calculated from the Eadie-Scatchard plot for DL-LysOMe: K_i = 18.81 mM.

In conditions similar to those used for L-LysOMe and DL-LysOMe hydrolysis study, we determined for D-LysOMe a maximal initial velocity V = 0.044 mM · min^{-1} and a Michaelis constant K_m = 32.66 mM.

3. Modification of the apparent optimal pH of immobilized trypsin

As reported above, the higher the pH, the more important the spontaneous hydrolysis of DL-LysOMe. Enzymic hydrolysis of DL-LysOMe has thus been performed at pH 5.5 because spontaneous hydrolysis may be neglected at this pH value. On the other hand, the optimal pH of immobilized trypsin is equal to 6.0.

It would thus be of interest to lower the apparent optimal pH of immobilized trypsin. Goldstein and Katchalski [8] have shown that this may be achieved by immobilizing an enzyme on a positively charged support. In the case of Spherosil, such a result may be obtained by co-grafting aminosilane and DEAE-silane onto the support. It is thus possible to prepare a support which presents available amino groups for glutaraldehyde activation and positively charged DEAE groups.

TABLE I

EFFECT OF GRAFTING OF POSITIVELY CHARGED GROUPS ONTO SPHEROSIL ON IMMOBILIZED TRYPSIN ACTIVITY

Initial amount of silane (g/100 g of Spherosil)		Amount of immobilized trypsin (mg/100 mg of support)	Activity (μmol · min^{-1})		
DEAE-silane	Amino-silane		pH 5.0	pH 5.5	pH 6.0
1	15	7.6	13.6	29.6	42.8
12	9	7.0	16.2	35.4	37.8
12	2.7	5.4	9.5	17.3	13.1

For this purpose, Spherosil was allowed to react with a silane solution in boiling xylene containing either only γ-aminopropyl trimethoxysilane or a mixture of NH_2-silane and DEAE-trimethoxysilane. The functionalized supports were activated with glutaraldehyde and used for trypsin immobilization. The activity of immobilized trypsin was determined using a 1.5 M DL-LysOMe solution at 30°C and at pH 5.0, pH 5.5 and pH 6.0. Table I shows that the amount of trypsin coupled onto the support is decreased by decreasing the aminosilane quantity. This may be a consequence of the lowered amino group content of silica. Furthermore, when the positive charge of Spherosil is increased by increasing the DEAE-silane: aminosilane ratio, there may be a repulsion of positively charged trypsin molecules (the isoionic point of which is equal to 10.1 [9]) by the positively charged support. As it can be seen in Table I, the introduction of DEAE groups (DEAE-silane to aminosilane ratio = 12 : 9, w/w) results in an important increase in the activity of immobilized trypsin at pH 5.0 and, particularly, at pH 5.5. At pH 5.5, the specific activity of immobilized trypsin is increased of 30%. On the other hand, when a higher DEAE-silane : aminosilane ratio is used (12 : 2.7, w/w) the activity is lowered at every pH.

Discussion

The aim of this work was to investigate the possibility of achieving optical resolution of DL-lysine by using the stereospecific action of trypsin on a DL-lysine ester, in view to obtain L-lysine and D-lysine ester.

The DL-lysine ester involved in optical resolution via trypsin esterasic activity must fall in with two conditions: (1) it must be very easy to prepare, particularly for large scale operation, and (2) it must be hydrolyzed by trypsin at as high a rate as possible. These two conditions made us take DL-lysine methyl ester as substrate. In fact, this derivative is easier to synthesize and more readily hydrolyzed than, for example, DL-lysine butyl ester.

The spontaneous chemical hydrolysis of DL-LysOMe, which would lower the optical purity of the product, may be neglected when operating at pH 5.5 and 30°C.

Enzymic hydrolysis of DL-LysOMe was studied with free and immobilized trypsin. Trypsin was fixed onto amine Spherosil activated with glutaraldehyde, for the action of which we proposed a mechanism [10]. The optimal pH is 5.8 for free trypsin. Optimal values of 5.8 [11], 6.0 [12] and 6.5 [13] have

been reported for trypsin acting on L-LysOMe. When trypsin is immobilized, its optimal pH is shifted towards pH 6.0. This may be a consequence either of the presence of negatively charged silanol groups at the silica surface, or, more probably, of a decrease of the number of positively charged amino groups of the trypsin molecule resulting from the immobilization reaction [14].

The Michaelis constant has been found equal to 8.25 mM and k_{cat} to 1680 min^{-1} at pH 5.5 and 30°C, for free trypsin acting on L-LysOMe. When DL-LysOMe is used as substrate, the corresponding Eadie-Scatchard plot gives a straight line, the intercept of which with the $v/[S]$ axis is the same as the intercept of the plot obtained with L-LysOMe as substrate (Fig. 2). This may be accounted for by the competitive inhibitory effect of D-LysOMe. It was possible to determine, from the Eadie-Scatchard plot for DL-LysOMe, the inhibition constant of D-LysOMe: K_i = 18.81 mM.

As reported for N^{α}-acetyl-D-lysine methyl ester [7], D-LysOMe is hydrolyzed by trypsin, but at a very much less rate than L-LysOMe: the initial hydrolysis rate of L-LysOMe is 327 times higher than the initial hydrolysis rate of DL-LysOMe. The hydrolysis of the D-form may thus be neglected when compared to the hydrolysis of the L-form, and trypsin action may therefore be considered as stereospecific.

The pH of trypsin action has been set at pH 5.5 in view to avoid DL-LysOMe spontaneous hydrolysis. We thus tried to increase enzymatic activity at this pH by decreasing the apparent optimal pH of immobilized trypsin, by modifying its electrostatic microenvironment. This was achieved by simultaneously grafting onto Spherosil an aminosilane and DEAE-silane: amino groups were activated with glutaraldehyde and used for enzyme coupling, while DEAE groups induce a pH gradient by repelling protons from the support surface. For that reason, pH is increased in the enzyme microenvironment [8]. So, while the bulk of substrate is kept at pH 5.5, trypsin acts at a higher pH and is thus more active. A DEAE-silane and aminosilane mixture (12 : 9, w/w) was grafted onto Spherosil. When trypsin is coupled via glutaraldehyde on such a support, its specific activity at pH 5.5 undergoes a 30% increase (Table I). Nevertheless, when aminosilane quantity is further decreased, i.e. for a DEAE-silane : aminosilane ratio of 12 : 2.7 (w/w), the activity of immobilized trypsin is decreased at every pH. This is a consequence of the decreased amount of coupled enzyme, but it may also be ascribed to charge-charge interactions between the enzyme molecule groups and DEAE groups. Besides, there may be a repulsion of the positively charged DL-LysOMe substrate by the positively charged support, resulting in a lowered DL-LysOMe concentration in the enzyme microenvironment.

In conclusion, the stereospecific action of trypsin on DL-LysOMe allows optical resolution of racemic DL-lysine. L-lysine production from chemically synthesized DL-lysine is thus possible. Continuous DL-LysOMe optical resolution may be achieved in a continuous reactor packed with immobilized trypsin. The efficiency of such a reactor is under study in our laboratory.

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