THE HYDROLYSIS OF PENICILLIN G TO 6-AMINO PENICILLANIC ACID BY ENTRAPPED PENICILLIN ACYLASE

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Penicillin acylase was extracted from *Escherichia coli* strain ATCC 9637, purified and entrapped in celluose triacetate fibres. The enzyme was immobilized by trapping it, as a finely dispersed aqueous phase, in yarns of cellulose triacetate by wet spinning. The kinetics of penicillin G hydrolysis were studied by use of several insoluble preparations with different activity content. In an attempt to evaluate the possible industrial application of the process, the performance of an entrapped acylase reactor was investigated.

Enzymes can be used to modify therapeutic agents, particularly antibiotics. One of the best known example is the conversion of benzylpenicillin to 6-aminopenicillanic acid (6-APA) by penicillin acylase.[1~3]

6-APA can be converted to semisynthetic penicillins, which exhibit several advantages over the parent penicillin G.

The conversion of penicillin G to 6-APA may be carried out either by a microbiological route or by a chemical route. The microbiological route may be replaced by an enzymatic route by using an insoluble form of penicillin acylase. Several methods have been used to prepare insoluble derivatives of enzymes.[4] Only a few papers dealing with penicillin acylase insolubilization have appeared.[5] For the present studies a physical entrapment of penicillin acylase into cellulosic fibres[6] was chosen.

By stirring a polymer organic solution with an enzymic aqueous solution an emulsion is obtained. The emulsion is then extruded through a spinneret into a coagulating bath. The enzyme remains entrapped as an aqueous solution within the great number of cavities formed in the fibres.

Experimental

Analytical Methods

The method of LOWRY *et al.*[7] was used for protein determination.

The enzymic activity of penicillin acylase was assayed by measuring the amount of 6-APA produced with the hydroxylamine method of BATCHELOR *et al.*[8] The incubation mixture contained 2 % penicillin G in 0.1M phosphate buffer, pH 8.5. The incubation temperature was 37°C. An enzyme unit is defined as the amount of enzyme required to hydrolyze 1 μ-mole of penicillin G in 1 hour under assay conditions.

Alternatively a pH-stat method was used by determining the initial NaOH consumption in a reaction mixture containing 2 % penicillin G in 0.01M phosphate buffer, pH 8.5.

The enzymatic activity of penicillin acylase-fibres was determined according to the following procedure: 10 g of wet fibres were placed parallelly to the axis of a silicon tube (70×1.2 cm) and fixed at the two extremities. The fibres were washed three times with a total volume of 1,500 ml of 0.01 M phosphate buffer.

A 4 % penicillin G solution (500 ml) in 0.01 M phosphate buffer, pH 8.5 was continuously recycled at a flow rate of 200 ml/min. The temperature was kept constant at 37°C. The initial NaOH consumption gave the corresponding value of enzymic activity. The enzymic activity is expressed as units per g of dry fibres.

Starch-gel electrophoresis of enzyme preparations was carried out by using the normal procedure. A tris-citric acid buffer, pH 8.5, was used.

Thin-layer chromatography of hydrolyzed mixture was carried out on Silica gel with the solvent system: *n*-butylacetate-glacial acetic acid-water (1:1:0.05). The spots were visualized by spraying the plates with a 30 % solution of NaN_3 in aqueous 0.1 N iodine.

Penicillin Acylase Isolation

Escherichia coli ATCC 9637 was fermented under submerged aerobic conditions; the composition of the medium expressed as g/liter was as follows: meat extract, 10; peptone, 10; NaCl, 5; phenylacetic acid, 2. The pH was adjusted to 7.0 and the temperature kept at 24°C. After 20 hours the cells were harvested by centrifugation, resuspended in 0.01 M tris-HCl buffer, pH 8.5, disrupted by recycling two times through a Manton-Gaulin homogenizer operated at 700 kg/cm². Hexadecylpyridinium chloride (0.4 %) was added to the suspension and the pH adjusted to 4.5.

The temperature was kept constant at 37°C. After 3 hours the insoluble material was removed by centrifugation through an Alfa-Laval LAB 102B-20 centrifuge. From the clear supernatant, the penicillin acylase was recovered by ammonium sulfate precipitation (65 % saturation).

Preparation of Entrapped Penicillin Acylase

The standard method described in a previous paper[6] was used. A solution of 27 g of cellulose triacetate in 200 ml methylene chloride at room temperature was prepared. To this solution a 35 ml enzyme solution in phosphate buffer, pH 8.0, containing glycerol, was added dropwise under gentle stirring. Stirring was continued for 30 minutes at 400 r.p.m. and the emulsion obtained was extruded through a spinneret into a coagulating bath containing toluene. The fibres obtained were vacuum-dried in order to eliminate methylene chloride and toluene.

Penicillin Hydrolysis with Acylase-fibres

The cellulosic fibres containing penicillin acylase were placed in a jacketed column parallelly to the axis and fixed at the two extremities. The reactor was connected by means of silicon tubes to a pump. The hydrolysis of penicillin G was carried out at 37°C by continuously recycling the antibiotic as a solution in 0.01 M phosphate buffer, pH 8.0, until the desired conversion value was reached. The pH was maintained at 8.0 through automatic addition of NaOH.

Results and Discussion

Enzyme Purification

The results obtained in the purification of the acylase according to the procedure described in the analytical methods section are shown in Table 1.

The purification procedure appears to be quite simple and can easily be adapted to large-scale isolation of the enzyme. In order to investigate the influence of the degree of purification on the performance of the entrapped acylase, the enzyme was further purified by chromatography through a DEAE-cellulose column. A typical elution profile is shown in Fig. 1. Specific activity of 1,000 was obtained and the electrophoresis showed a major peak and two minor peaks.

Activity of Penicillin Acylase-fibres

Fibres containing different amounts of enzyme were prepared with the aim of evaluating the influence of the amount of entrapped activity on the activity displayed by the fibres, *i.e.* the "degree of utilization" of the enzyme by the fibre, as a function of its enzyme content. The entrapped activity is defined as the amount of enzyme put into the emulsion and is expressed as

Table 1. Purification of penicillin acylase.

Step	Volume (liter)	Total protein (g)	Total units (μ moles/hr)	Specific activity (μ moles/hr/mg)	Recovery (%)
Bacterial suspension	14.0	—	13.34×10^6	—	100
Disrupted suspension	13.95	550	11.05×10^6	20.2	82.9
Supernatant	14.1	126.9	8.52×10^6	67.0	63.8
Ammonium sulfate pptn. (redissolved)	1.0	81.5	8.2×10^6	100.6	61.5

Table 2. Activity displayed by penicillin acylase-fibres as a function of entrapped activity.

Preparation No.	Entrapped activity (units/g dry fibres)	Activity measured (units/g dry fibres)	Specific activity of entrapped enzyme (units/mg protein)
PA/6	600	510	33
PA/4	1,000	590	21
PA/1	2,100	980	21
PA/11	2,400	985	90
PA/10	4,750	1,420	90
PA/9	7,900	1,870	90
PA/12	10,300	2,060	90
PA/13	10,000	2,400	1,000

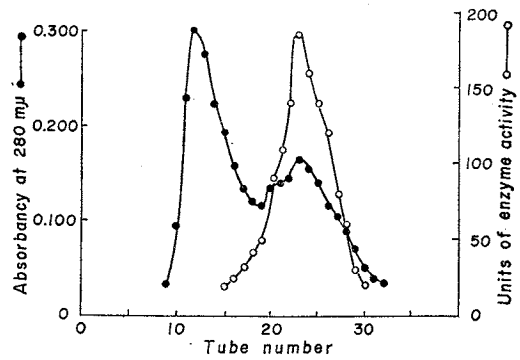
Table 3. Experimental data obtained in a typical penicillin hydrolysis experiment by using penicillin acylase trapped in fibres.

Time in minutes	NaOH consumption (ml NaOH 3.9 N)	Total penicillin G (moles)	Total 6-APA (moles)
0	0	1.875	0
29	100	1.470	0.368
66	200	1.090	0.716
117	300	0.728	1.090
186	400	0.321	1.445
233	445	0.161	1.585
310	466.5	0.049	1.655

Table 4. Effect of flow-rate on the rate of penicillin G hydrolysis by acylase-fibres reactor.
Recycling volume : 5.5 liters
Penicillin G, K salt: 720 g

Flow-rate (liter/hr)	Time for 85% hydrolysis (min.)	Time for 25% hydrolysis (min.)
46.5	255	58.5
71.0	270	52
100.0	214	43
216.0	213	40.5
260.0	196	36
309.0	192	37
360.0	190	36

Fig. 1. Elution pattern of penicillin acylase from DEAE-cellulose.
A column (1×30 cm) was equilibrated with phosphate buffer, pH 8.0, the flow rate was 12 ml per hour, and fractions of 3 ml per tube were collected.



units per gram of cellulose triacetate. This was a realistic definition since it was shown that no denaturation of enzyme by the solvents occurred during spinning. The results are shown in Table 2.

The data show that the percentage of entrapped activity displayed by the fibres was decreased by increasing the concentration of the enzyme. Apparently a fraction of the entrapped enzyme is not saturated by the substrate because of diffusion limitations. It is also noteworthy that the purity of the enzyme does not appreciably affect its performance inside the fibres.

Effect of Temperature

The activity of fibres containing 2,100 penicillin acylase units per g of dry polymer was investigated as a function of the temperature.

Table 5. Continuous penicillin hydrolysis by the acylase fibres reactor. Substrate solution: 4% Penicillin G, K salt, in pH 8.5 phosphate buffer.

Buffer molarity (m/liter)	Flow-rate (liter/hr)	Outlet pH	% Hydrolysis
0.1	2.78	6.4	80.0
0.2	2.78	7.05	89.6
0.2	0.90	6.7	93.4

From ARRHENIUS plot an activation energy of 12,500 cal/mole can be calculated, while for the free enzyme a value of 13,750 cal/mole was obtained. The lower activation energy found for the entrapped enzyme was interpreted in terms of diffusion limitation. To explain this finding, the WHEELER-THIELE[9,10] analysis for simultaneous diffusion and chemical reaction in a porous catalyst may be used. The same approach has been used also to correlate the sucrose inversion data in resin particles.[11] According to the WHEELER-THIELE model an arithmetic average of chemical and diffusion processes activation energy is expected.

In practice the penicillin acylase-fibres behaved as a better catalyst at low temperature than the free enzyme. This could be an advantage for industrial hydrolysis of penicillin G taking into account the instability of the antibiotic molecule.

Stability of Acylase-fibres

The acylase-fibres, under storage conditions, at 4°C, were found to be quite stable. No loss of activity was detected after 6 months.

Under catalytic conditions, at 37°C, after an initial drop due to leakage of not well-entrapped enzyme, the activity remains practically constant, at the value corresponding to 80 % of the initial one, for 5 months.

Like the free enzyme, the entrapped penicillin acylase is quite sensitive to alkaline pH.

No loss of activity by casual bacterial contamination was found, because the entrapped enzyme is protected against proteolytic attack.

Bench-scale Experiments

By using a 7.6×95 cm column containing 1 kg of penicillin acylase fibres (10,000 units/g of cellulose triacetate), the hydrolysis of 12 % penicillin G solutions was carried out according to the described procedure. The NaOH consumption was recorded and penicillin G and 6-APA were determined. The results of a typical kinetic are shown in Table 3.

Analysis of the experimental data indicated that the sum of total penicillin G plus total 6-APA present in the mixture decreased during hydrolysis. Apparently a chemical decomposition of penicillin G and 6-APA occurred. At the end of the hydrolysis the loss was about 9 %.

In order to obtain a high yield of conversion of penicillin G to 6-APA, it is necessary to reduce the hydrolysis time. Since the technology of enzymatic fibres allows very large amounts of enzyme to be entrapped, it is possible to obtain very active fibres. By using these fibres a remarkable reduction of the hydrolysis time was achieved and a conversion yield higher than 90 % was reached.

Effect of the Flow-rate on the Reaction Rate

The kinetics at various flow-rates were investigated. The results are shown in Table 4.

As expected, the effect of flow-rate was greater in the early part of the kinetic, where the NaOH consumption was higher (*i.e.* the pH drop between the bottom and the top of column was larger).

A further advantage of increasing the flow-rate was that the diffusion problems were overcomed.

It was noticed that even at relatively high flow-rates the fibres showed a quite good mechanical resistance.

Continuous Penicillin Hydrolysis

Some experiments were conducted in order to establish also the feasibility of a continuous penicillin hydrolysis (*i.e.* only one pass of antibiotic solution through the column), without pH control. The results are shown in Table 5.

In these experimental conditions the activity of the fibres dropped remarkably because, evidently, a large amount of the enzyme was not operating at its optimum pH. Higher activities were obtained by increasing the buffer molarity.

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