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Prodution and Properties of the Insoluble Penicillinase from *Streptomyces*

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放線菌이 分泌하는 不溶性 Penicillinase

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Abstract

A *Streptomyces* sp. strain AS-727 which was capable of producing penicillinase, was isolated from soil. The enzyme production was affected by the carbon and nitrogen sources added. Among them so far tested, glucose (or maltose) and sodium nitrate increased the enzyme production. And the amount of enzyme prodced reached maximum in 4 days cultivation.

The optimla pH and temperature of the penicillinase was between pH 6.0 to 8.0 and 40°C respectively. The stabel pH range of the enzyme was stable at 40°C, but it lost about 30% and 40% of the the activity respectively when it was treated at 60°C and 80°C for 60 minutes.

The activity of the enzyme was inhibited by Zn^{++}, but Ag^{+}, Co^{++}, Mn^{++}, Ca^{++}, Pb^{++} did not affected enzyme activity.

Peculiarly, the enzyme protein precipitated by freezing or addition of ammonium sulfate, urea, sodium chloride and some organic solvents as etanol, methanol, acetone was not dissolved in deionized water or any buffer solution.

Intorduction

Penicillinase (β-lactamase, penicillin amide-β-lactamhydrolase, EC3, 5, 2, 6) has been many investigated. Since Abraham and Chain[1] reported the penicillin-destroying activity in extracts of *Escherichia coli* and the enzyme activity was presumed to have a primary role for the high resistance of the organisms to penicillins, there have been many reports on the synthesis of penillinase by many gram-positive[2,3] and gram-negative bacteria[4,5,6] and *Actinomyces*[7,8].

Up to the present, the studies for enzymatic properties, mechanisms for inducible production [9~11], structure and active center[12,13], immunochemistry of the penicillinase[14~16] have been

carried out.

We obtained insoluble penicillinase of different type from known enzymes. This paper deals with the isolation and culture conditions for producing the penillinase and some properties of the enzyme.

Materials and Methods

Media; Isolation medium consisted of glucose 1%, pepton 0.2 %, agar 1.8 %. Culture medium consisted of glucose 1.0 %, $NaNO_3$ 0.2 %, K_2HPO_4 0.01 %, $MgSO_4 \cdot 7H_2O$ 0.01 %. After sterillization, pH of the medium was 5.4.

Isolation of the organism; The penicillinase producing *Streptomyces* sp. strains were isolated from soil according to the microiodometric method [17]. *Streptomyces* sp. AS-727 was selected as one of the most potential penicillinase producer, and used for the present study.

Culture; Using 1000 m*l*-Erlenmeyer flask containg 300 m*l* of medium, the strain AS-727 was stand-cultured at 30°C for 4 days.

Enzyme assay; The penicillinase activity was determined by microiodometric method[17] at 40°C and enzyme activity was described in relative activity. Used buffer was 0.1 M phosphate buffer (pH 7.0). Cell growth was expressed as milligrams of cell dry weight eqivalent of organisms per 10 m*l* of cultured broth.

Chemicals; The crystalline penicilline G (4000 units/mg) was purchased from Hoechst Remedia Ind. Co.. And other chemicals used were of reagent grade.

Preparation of the enzyme solution; To the cultured filtrate, solid ammonium sulfate was added to give a final saturation of 100%. After two hours, the suspension was centrifugated at 12000 rpm for 10 minutes. Obtained precipitates were collected and carried out dialysis against deionized water at 4°C for two days with three changes of water. Then soluble part was discarded and remaining precipitates were homogenously suspended in 0.1 M phosphate buffer (pH 7.0), and it was used as enzyme solution.

Results and Discussion

Effect of carbon source on enzyme production

Adding various sugar as carbon source in the concentration of 1 % to the medium, penicillinase producing strain AS-727 was cultured, and the final pH and the enzyme activity were measured. The results are summerized in Table 1. All sugars so far tested supported the cell growth, however, the most suitable sugar for enzyme production was glucose and maltose but xylose was ill in growth and enzyme production.

Table 1. Effect of Carbon Source on Enzyme Production.

The concentration of various carbon sources was 1 % in culture medium and initial pH was 5.4. The activity added glucose was set at 100.

Source	Final pH	Growth	Relative activity
s-Starch	6.2	##	44
Lactose	6.2	##	56
Maltose	6.8	##	108
Sucrose	6.6	###	44
Fructose	6.0	##	33
Glucose	6.2	###	100
Xylose	5.9	+	1
Lycerin	6.2	###	74

Effect of Nitrogen Source on the Enzyme Production

Effects of organic as well as inorganic nitrogen sources were examined (Table 2) using at the concentration of 0.2 %. It was found that only sodium nitrate appeared best for the production of penicillinase from the strain of AS-727, also urea and peptone enhanced considerably the enzyme production, whereas in the sodium nitrite containg medium the organism was not grown.

Time Course of Enzyme Production

We examined time dependent changes of the enzyme production, growth of cell mass, changes of pH of the medium with culturing the strain of AS-727 in 100 m*l* Erlenmeyer flask contained 20 m*l* of medium. After inoculation, the culture flasks were incubated at 30°C. As shown in Fig. 1, the

Table 2. Effect of Nitrogen Source on Enzyme Production.

The concentration of various N-sources was 0.2 %, and initial pH was 5.4. The activity added peptone was set at 100.

N-source	Final pH	Growth	Relative activity
peptone	7.2	###	100
yeast ext.	6.0	##	85
asdragine	6.2	##	19
$(NH_4)_2HPO_4$	6.0	##	15
$(NH_4(_2SO_4$	5.4	##	19
NH_4Cl	5.4	##	15
$(NH_2)_2CO$	7.4	+	120
$NaNO_3$	6.6	##	145
$NaNO_2$	6.4	−	0

productivity of the enzyme reached to a maximum level after four days the incubation was started. Cell growth gained maimum stationary level after seven days cultivation followed by a slight decrease. The pH of medium raised slowly after two days.

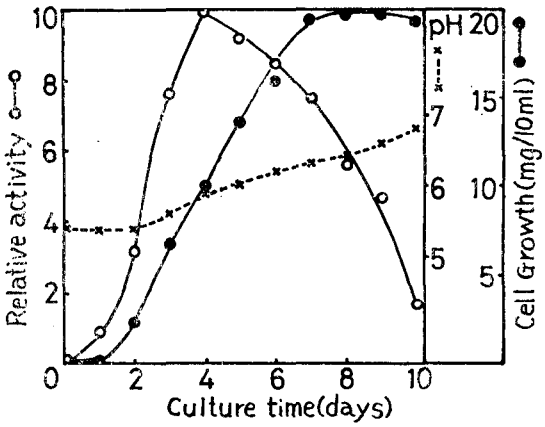

Fig. 1 Time Course of Enzyme Production.

The composition of the medium is discribed in "Materials and Methods" and the cultivation process was carried out under the following conditions: 30°C, stationary, 20 m*l* medium in 100 m*l*-Erlenmeyer flask.

Effect of pH

The penicillinase produced by the strain of AS-727 hydrolyzed penicillin G to penicilloic acid. The activity of aqueous suspension of the enzyme was assayed at different pH, using following buffer systems; McIlvaine buffer (0.1 M citrate~0.2 M Na_2HPO_4, pH 2.0 to 8.0), Clark and Lube buffer (0.1N NaOH~0.1N Borate KCl, pH 8.0 to 10.0).

Other conditions were the same as those of standard assay method. As shown in Fig. 2, the optimal pH range was between 6.0 to 8.0. But below pH 3.0 and above pH 10.0, the enzyme showed no activity.

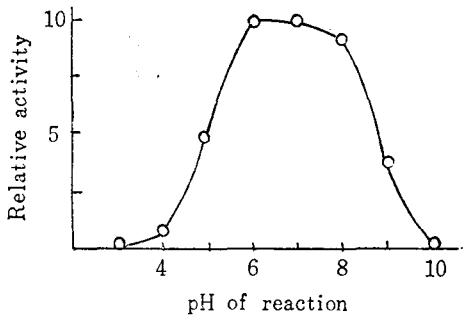

Fig. 2 Effect of pH on Enzyme Reation.

The enzy me activity was assayed in the buffer of various pH values at 40 °C. pH 2.0~8.0: McIlvaine buffer 8.0~10.0: Clark and Lube buffer. The activity at pH 6.0 was set at 10.

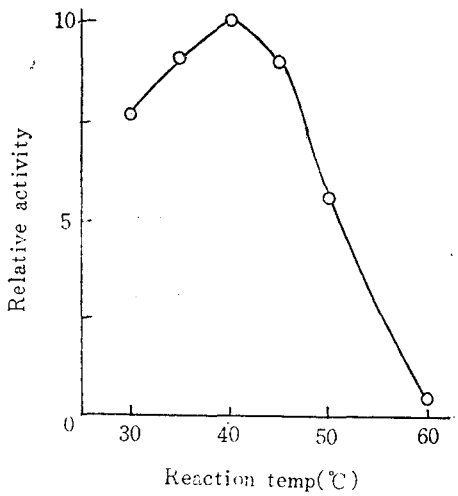

Fig. 3. Effect of Temperature on Enzyme Reaction.

Activities of the penicillinase were measured in M/10 phosphate buffer (pH 7.0) at various temperatue. The activity at 40°C was set at 10.

Effect of Temperature

The penicillinase activity of suspension was assayed at different temperature range from 30°C to 60°C. The assay was carried out by the standard method at pH 7.0. As shown in Fig. 3, the maximum penicillinase activity was revealed at around 40°C.

Effect of pH on sfability of Penillinase

The enzyme suspension was incubated in buffer (systems were the same as those described in the test of optimal pH) of different pH at 37°C for 60 minutes and at 4°C for days, and then residual activity was measured by standard assay method. As illustrated in Fig. 4, the enzyme was stable in wide ranges beween pH 4.0 to 9.0.

As a result of this test, we found that this enzyme was very stable in pH variation.

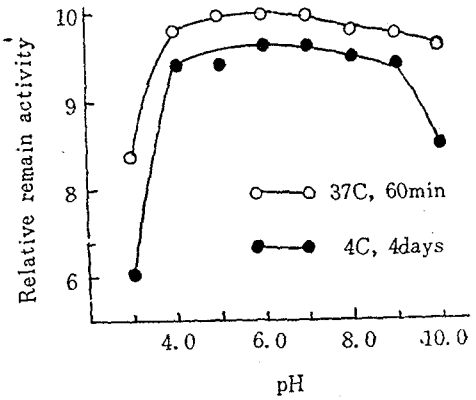

Fig. 4. Effect of pH on Stability of Enzyme.
Suspended enzyme was treated in the various pH values at 30°C for 60 minutes and 4°C, for 4 days. pH 3.0–8.0: McIlvain, pH 8.0–10.0: Clark and Lube buffer. The activity remaining after treatment at pH 6.0 was set at 10.

Thermostability of Ensyne

Aqueous suspension of the enzyme (pH 5.6) was treated at 40, 60 and 80°C for various time 0 to 60 minutes and residual activity was assayed at pH 7.0. As illustrated in Fig. 5, the enzyme was stable at 40°C. And when it was incubated at 60C and 80°C for 60 minutes, the enzyme lost its activity about about 30% and 40% respectively.

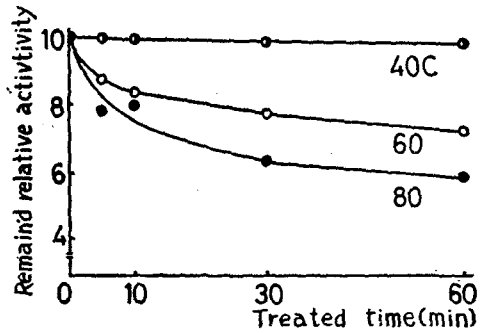

Fig. 5. Effect of Temperature on Stability of Enzyme.
Suspension of the enzyme was treated at 40, 60, 80°C for various time. The remaining activity was assayed at 40°C and pH 7.0. And the activity not treated was set at 10.

Therefore the enzyme was stable against heating compared with the *Streptomptomyces albus* G penicillinase[8] which was completely inactivated by heating at 60°C for 5 minutes.

Effect of Metal Salts on Activity of Penicillinase

To investigate the effect of metal salt on the enzyme activity, various metal salts, $AgNO_3$, $PbNO_3$, $ZnCl_2$, $CoCl_2 \cdot 6H_2O$, $MnCl \cdot 24H_2O$, $MgSO_4 \cdot 7H_2O$ and $CaCl_2$ were added to the reaction mixture at the final concentration of 1 mM. As shown in Table 3, no significant effect can be realized with any of the metal salts tested but considerable inhibition of the enzyme was seen in the presence of $ZnCl_2$.

Table 3. Effect of Metal Salts on the Enzyme Activity.
The concentration of metal salts in the rection a mixture was 1 mM, and the activity not added was set at 100.

Metal salt*	Relative activity
None	100
$AgNO_3$	83
Pb-acetate	89
$CoCl_26H_2O$	112
$MnCl_24H_2O$	94
ZnC_2l	77
$MgSO_47H_2O$	98
$CaCl_2$	100

* : Concentration of metal=1 mM

Effect of Concentration of $ZnCl_2$ on Activity

The reaction mixtures containing various concentration from 10^{-1}M to 10^{-1}M were incuvated at 40 °C for 10 minutes. As illustrated in Fig. 6, the enzyme was inhibited about 20 % by addition of zinc chloride in the concentration of 10^{-3} and inhibited more than about 95 % in the high concentration above 10^{-2}M.

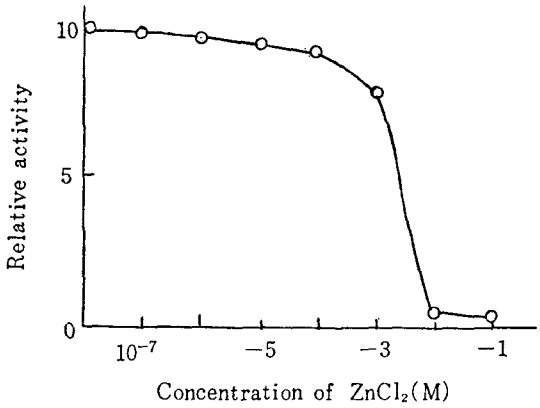

Fig. 6. Effect of Concentration of $ZnCl_2$ on Activity.
The concentration of $ZnCl_2$ in the reaction mixture was 10^{-1}M to 10^{-7}M. And the enzyme activity was assayed at 40°C for 10 minutes. The activity not added was set at 10.

Denaturating to Insoluble Enzyme:

Once the enzyme protein in the cultured broth was prectipitated by freezing, addition of ammonium sulfate, urea, sodium chloride or some organic solvents as methanol, ethanol and acetone, it did not dissolved again as shown in Table 4. This property of the enzyme was different from the other penicillinase reported earlier.

Table 4. Denaturating to Insoluble Enzyme.
Once the enzyme protein in cultured broth was precipitated with freezing, $(NH_4)_2SO_4$, urea, NaCl, MeOH, EtOH, acetone, the formed precipitate was insolube.

solution ⟶ ppt ⟶ insoluble	
↑ factor	
freezing	+
ammoniumsulfate	+
urea	+
sodiumchloride	+
org. solvent (MeOH, EtOH, acetone)	+

要 約

特異하게도 일단 어떤 原因으로든지 變性이 일어나서 沈澱이 形成되고 난 後에는 그 變性으로 因하여 다시 溶解되지 않는 penicillinase를 生成分泌하는 *Streptomyces* 屬 菌株 AS-727을 토양으로부터 얻었으며, 이 菌의 酵素生産性과 이 酵素의 一般的性質을 檢討한 結果는 다음과 같다.

1) 本 酵素의 生産을 爲해서는 窒素源으로서 sodium nitrate, 炭素源으로서 glucose 나 maltose를 使用하여 4日間 培養하는 것이 바람직했으며

2) 精製는 ammonium sulfate로 完全飽和시켜 生成된 沈澱을 遠心分離로 모아 그것을 透析해서 粗精製酵素를 얻는데, 이때 透析할 때 沈澱은 溶解되지 않는다.

3) 本 酵素의 最適 pH는 中性附近 (6.0~8.0)이며 最適溫度는 40°C 近處이고,

4) 安定 pH 範圍는 4.0~9.0으로서 比較的 넓었으며 熱에 對해서는 40°C에서는 安定했으며 60°C와 80°C에서 60分間 處理하면 각각 約 30%와 40%가 失活되었다.

5) 本 酵素의 活性에 있어서 zinc chloride가 10^{-3}M 濃度에서 約 20%, 10^{-2}M 以上의 高濃度에서는 95% 以上을 阻害하였다.

6) 그리고 凍結이나 各種 鹽類 또는 有機溶媒로 일단 形成된 酵素蛋白沈澱은 다시 溶解되지 않는 特異한 性質을 가졌다.

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