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Optimization of Penicillin Amidase Production and A Simplified Enzyme Assay Method

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페니실린 아미다제 생산의 최적 조건 및 간이화 한 효소 정량 방법에 대한 연구

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ABSTRACT

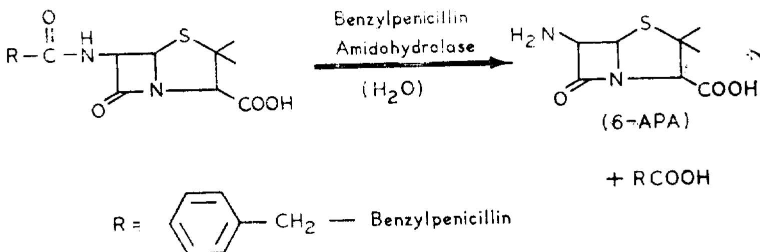
Penicillin amidase (EC 3.5.1.11) was produced by a mutant strain of *Bacillius megaterium* ATCC 14945. Hydroxylamine assay method for the determination of 6-APA was modified by using "HCl addition technique" in order to simplify the time consuming original assay method without sacrifice of accuracy. Using the new mutant strain, the effects of fermentation conditions on enzyme production were studied.

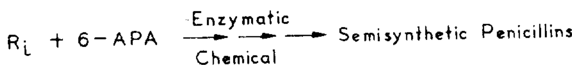
INTRODUCTION

The penicillin molecule can be hydrolyzed to yield 6-aminopenicillanic acid (6-APA) and its side chain carboxylic acid by penicillin amidase. This amidase has great commercial importance, because 6-APA is the starting material for the synthesis of new semi-synthetic penicillins (Fig. 1).

Recently, there has been some renewed interest in further study and development of the enzymatic process for the production of 6-APA because of problems related to possible carcinogenicity of dimethylaniline that has been used as the solvent in chemical hydrolysis of penicillin.

Penicillin amidases hydrolyze preferentially benzyl penicillin or phenoxymethyl





R_i = New side chains

Fig. 1. Enzymatic hydrolysis of penicillins by penicillin amidase.

penicillin according to their substrate specificities. Benzyl penicillin amidase is mostly found in bacteria, [1~15] and penicillin V amidase is usually found in molds and actinomycetes [5,9,12,13,16~23] but many exceptions to these general observations exist. [12,13,24] Since penicillin amidase activity was first described by Sakaguchi and Murao[16] in 1950, there have been many reports on this enzyme and its applications. However, the effect of practical process variables on the productivity and yield of this enzyme has not yet been fully elucidated and reported.

In view of scarcity of practical information on this enzyme we are far from being able to control the microbial metabolism to overproduce this enzyme. The productivity of microbial enzyme can be increased in general by genetic manipulation and control of environmental conditions. [25,26] Especially, enzyme production by microorganisms is sensitive to conditions of culture environment. If we are to search for the optimal culture condition for the maximal enzyme productivity for a superior mutant, we should understand at least part of the microbial physiology of the new mutant strain. Such a study and understanding will enable us to overproduce the desired enzyme by reasonable genetic and environmental control. In view of this, using known benzyl penicillin amidase producer, a mutant strain of *Bacillus megaterium*, various fermenation conditions including medium composition, inducer, induction mode, and physicochemical variables are studied for the increase of benzyl penicillin amidase activity.

MATERIALS AND METHODS

1. **Microorganism.** A new mutant strain of *Bacillus megaterium* (ATCC 14945) was used in all experiments. A superior mutant was obtained by using combination of U.V. irradiation and chemical mutagenic agents.

2. **Fermentation.** Seed culture media[6] were inoculated from an agar slant and were cultivated aerobically (250rpm in rotary shaker) at 30°C for 24 hrs. The fermentation media consisted of carbon source, 1.0%; organic or inorganic nitrogen source, 2.5%; yeast extract, technical, 0.5%; phenylacetic acid or benzoic acid, 0.2% as K salt; and silicone antifoam B-emulsion (Dow Chemical Co., Midland, Mich.), 0.01%. The carbon source was sterilized separately. Fermentation media (50 ml) were adjusted to *p*H 6.5 before autoclaving.

Each 500 ml Erlenmeyer flask containing sterile fermentation media was inoculated with 5% seed culture and was shaken on a rotary shaker (250rpm at 30°C). After 48 to 72 hr of cultivation, the broth was harvested, treated with 0.2% (V/V) toluene, and centrifuged at 3,000rpm for 30 min or at 8,000 rpm for 10 min. This centrifuged supernatant was used as the source of crude enzyme.

3. **Measurement of cell growth.** The dry weight of twice-washed cells (dried at 85°C for 12 hr) was measured, its correlation with turbidity reading at 600nm with cell suspension was determined and a calibration curve was prepared therefrom. One optical density unit was found to correspond to 0.483g-cell/*l*.

4. **Qualitative determination of enzyme activity.** In order to identify the 6-APA formed during enzyme reaction, thin layer chromatography (TLC) on silica gel GF(Fischer) was performed according to Nara *et al.*[13]

5. **Enzymereaction.** The reaction mixture

consisted of substrate solution containing 10mg of potassium benzyl penicillin in 1ml of 0.1M borate buffer adjusted to pH 8.7, and 1ml of crude enzyme solution, and incubated at 40°C for 1hr. The enzyme activity was determined from the reaction rate measured.

6. Modified hydroxylamine assay with-out extraction steps. For the measurement of 6-APA in reaction mixture, hydroxylamine method[27,28] was modified to some extent. One ml of reaction mixture was pipetted into a test tube, followed by addition of 1ml of 0.8N HCl and allowed to stand at 25°C for 12 to 16 min. Then 5 ml of hydroxylamine reagent solution was added. After 10 to 15 min, 1ml of ferric ammonium sulfate solution (20% $Fe_2(NH_4)_2(SO_4)_4 \cdot 24H_2O$ in 2.4M H_2SO_4) was added, mixed well and after 5 to 7 min, absorbancy at 490nm was read. Hydroxylmine reagent was adjusted to pH7.4.

For the blank correction, enzyme solution and substrate solution were incubated separately before assaying. Hydroxylamine reagent was prepared by mixing the alkaline buffer (2.4% $CH_3COONa \cdot 3H_2O$ in 5.5 M NaOH), hydroxylamine hydrochloride (40% solution), distilled water, and absolute ethanol (1 : 1 : 4 : 4).

The reaction steps involved in this assay method are illustrated in Fig. 2. By acid treatment of the residual benzyl penicillin, penillic acid is formed but is not reactive with hydroxylamine while 6-APA and hydroxylamine react to give a hydroxa mate which in turn gives color complex with ferric ion.

RESULTS AND DISCUSSION

1. Improvement of assay method. It was found that potassium salt of benzyl penicillin is extremely unstable under acidic condition while 6-APA is stable. Fig. 3 shows that benzyl penicillin was converted completely to penillic acid within 8 to 10 min, whereas 6-APA remained unchanged. 12 min after the addition of 0.8N HCl solution, 6-APA was destroyed by less than 4%. By making use of this difference in relative stability, conventional hydro-

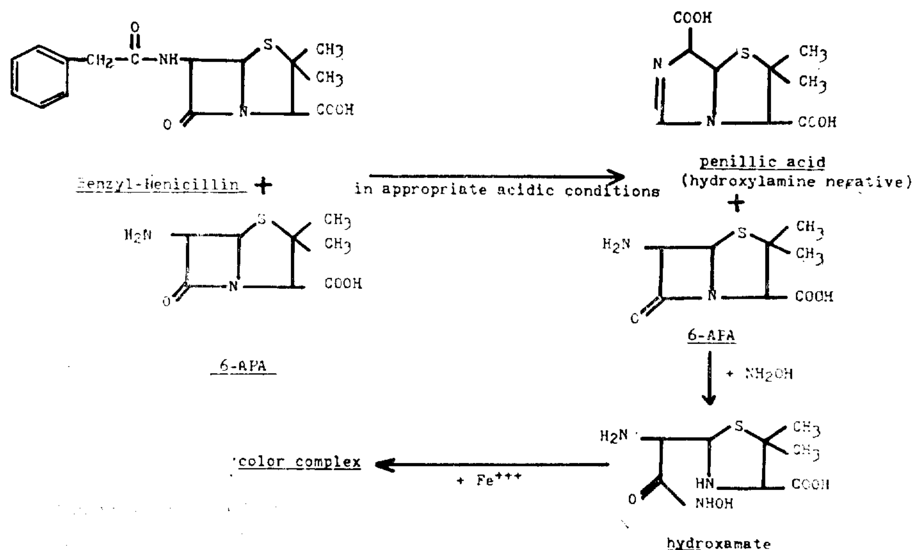
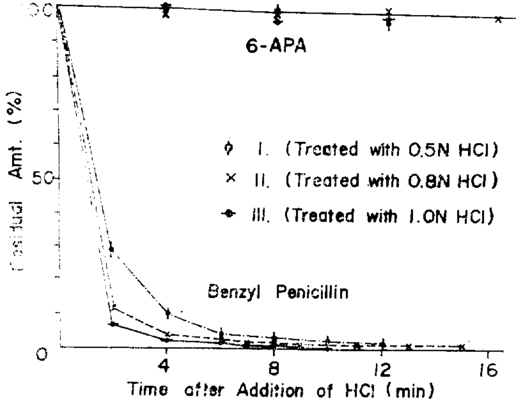


Fig. 2. Reaction steps involved in the modified assay method for penicillin amidase.



Fg. 3. Effect of acid treatment of 6-APA and benzylpenicillin.
1.0ml HCl solution was added to 1ml of 4mM 6-APA solution in borate buffer (pH8.7) and also to 1ml of substrate solution containing 5mg benzylpenicillin.

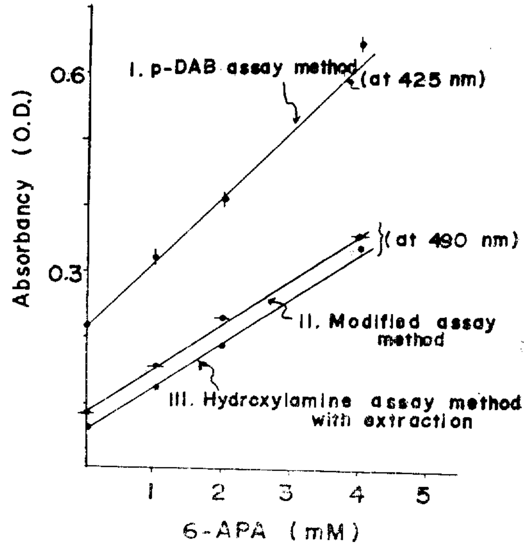


Fig. 4. Comparison of assay methods for penicillin amidase.
The slope represents the extinction coefficient and the intercept represents possible interference of components contained in the reaction mixture and the blank.

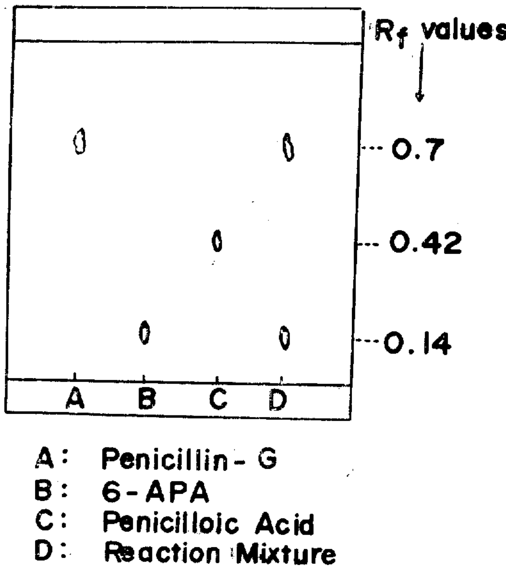


Fig. 5. Thin layer chromatographic analysis of reaction mixture.

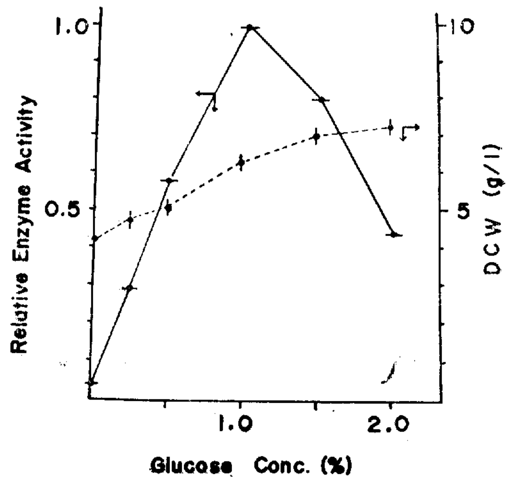


Fig. 6. Effect of glucose concentration on cell growth and enzyme yield.
Basal medium: Casitone 2.5%
yeast etxract 0.5%, PAA 0.2%

Table 1. Effect of carbon sources on enzyme yield.(a)

Carbon source (0.5%)	DCW(b) (g/liter)	Final pH	Activity (unit/ml)	Relative activity(%)
D-Glucose	6.05	8.8	5.63	100
D-Fructose	5.63	8.7	4.38	77.8
Sucrose	4.36	9.0	3.23	57.4
Glycerol	6.29	8.6	3.75	66.6
Maltose	5.46	9.1	2.61	46.4
Starch	5.35	9.2	2.40	42.6
Sodium citrate	4.71	9.3	2.10	37.3
Sodium acetate	4.35	9.3	1.12	19.9
Lactose	4.44	9.2	1.15	20.4
D-Mannose	4.38	9.1	0.18	3.2
D-Galactose	4.56	9.2	2.08	36.9
L-Sorbose	3.54	9.2	0.74	13.1

(a) Basal medium: Casitone 3%, PAA 0.2%
(b) Dry cell weight

xylamine assay[27,28] was modified.

Modified hydroxylamine method with "HCl addition technique" was found to be equivalent in terms of sensitivity and accuracy of the assay to the hydroxylamine method. The advantage of the modified method lies in the fact that it does not require tedious and laborious extraction with n-butyl acetate. These two methods showed nearly the same molar extinction coefficient, ϵ_M, of about $70M^{-1}cm^{-1}$(Fig. 4). The p-dimethylaminobenzaldehyde(p-DAB)[29,30] method showed slightly higher value than hydroxylamine methods. The signifi cant interference found in p-DAB method was caused by compounds with amino groups present in the crude enzyme solution. This assay was inferior to our modified assay method. Another advantage with our modified method is that this may be performed quite readily with automatic wetchemical analysers(*i.e.* Auto-Analyser), and is suitable for handling many samples manually or automatically in relatively short time.

2. Identification of 6-APA in reaction mixture. The product from reactionmixture was identified by the TLC as 6-APA (Fig. 5). No penicilloic acid (hydrolytic product of penicillin by penicillinase) was detected. The TLC data confirmed that the mutant did not produce penicilloic acid. Thus, the mutant is considered a good candidate for industrial application in production of penicillin amidase.

3. Effect of carbon source on enzyme production. Various carbon sources were tested for the enzyme production and the result was shown in Table 1. Among those tested, glucose was the best carbon source and fructose, glycerol, and sucrose gave 57—77% activity. Others were found to be poor carbon sources for both the cell growth and enzyme production.

As shown in Fig. 6, the final cell concentration was increased almost in parellel with the increase of the glucose concentration. However, enzyme activity showed a maximum at 1% glucose concentration. Too high a concentration of glucose was inhibitory to enzyme biosynthesis, possibly due to catabolite repression.

4. Effect of nitrogen source on enzyme production. Various nitrogen sources were

Table 2. Effect of organic nitrogen sources on enzyme yield period, (a)

Nitrogen source (3%)	DCW (g/liter)	Final *p*H	Activity (unit/m*l*)	Relative activity(%)
Casitone	5.24	8.4	4.89	100
Casamino acid, technical(b)	1.67	8.7	0	0
Casamino acid(b)	1.04	7.9	0	0
Bacto peptone	0.99	7.3	0	0
Bacto tryptone	4.12	8.5	2.31	47.2
Corn steep liquor (50% syrup)	7.33	8.2	0	0
Soy bean meal	−(d)	7.8	0	0
Yeast extract, technical	6.75	8.7	3.74	76.5
Bouillion (c)	2.02	7.6	0	0
Casein	1.40	7.3	0	0

(a) Basal medium: glucose 0.5%, PAA 0.2%

(b) Supplemented with 0.02% tryptophan.

(c) Bacto-peptone 1%+meat extract 1.5%+NaCl 0.5%

(d) Not measured

Table 3. Effect of supplementing yeast extract to the nitrogen source period (a)

Nitrogen source	DCW (g/liter)	Final *p*H	Activity (unit/m*l*)	Relative activity(%)
Casitone 3%	6.66	8.2	7.04	73.1
Y. Extract 3%	7.36	8.4	1.40	14.5
Casitone 2.5%+Y. Ext. 0.5%	7.03	8.3	9.63	100
Tryptone 2.5%+Y. Ext. 0.5%	7.14	8.3	3.89	40.4
Casamino acid 2.5%+Y. Ext. 0.5% (b)	7.20	8.3	4.87	50.6
NH_4Cl 2.5%+Y. Ext. 0.5%	3.16	7.3	7.50	77.9
Corn steep liquor 2.5%+Y. Ext. 0.5%	7.72	8.1	0.07	0.7
Peptone 2.5%+Y. Ext. 0.5%	3.54	8.0	0.02	0.2
Urea2.5%+Y. Ext. 0.5%	0.19	8.6	0.13	1.3

(a) Basal medium: glucose 1%, PAA 0.2%

(b) Supplemented with 0.02% tryptophan.

evaluated in order to find the best nitrogen source. As shown in Table 2, only casitone, tryptone, and yeast extract gave good yield of enzyme. Casamino acid and peptone did not provide good growth and enzyme production. This poor growth observed may be due to lack of growth factors and vitamins. To determine the possible effect of growth factors and vitamins the nitrogen sources were supplemented with 0.5% yeast extract primarily as growth factors (Table 3). The medium supplemented with yeast extract gave higher cell growth and resulted in 40% increase in enzyme production. When urea was used, the growth was very poor. Apparently, our mutant strain does not have the urease activity.

From the results of Tables 2 and 3, we can see an important tendency: casein hydrolyzates such as casitone, tryptone,

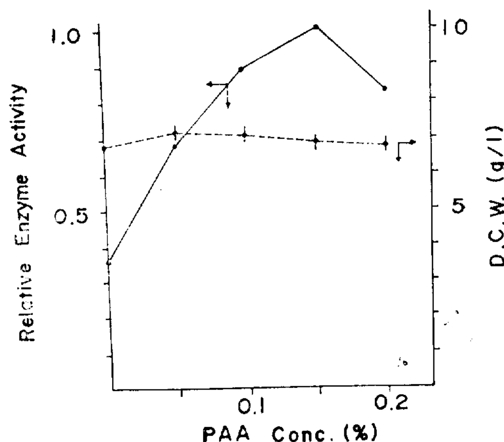


Fig. 7 Effect of inducer (PAA) concentration on the enzyme yield. (See the text for the experimental conditions)

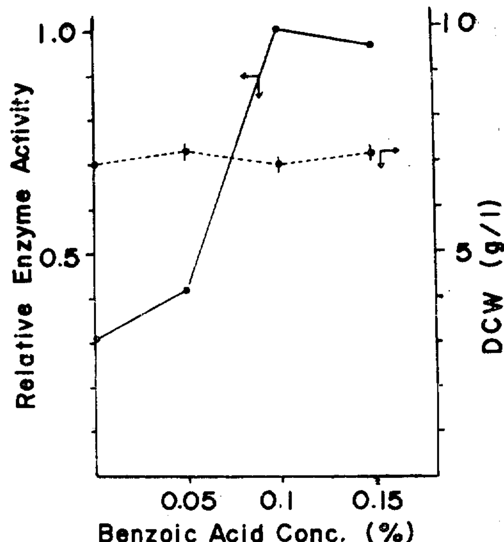


Fig. 8 Effect of inducer (Benzoic acid) concentration on the enzyme yield. (See the text for the experimertal condition)

Table 4. Effect of initial *p*H of medium on enzyme yield. (a)

Initial *p*H	DCW (g/liter)	Final *p*H	Activity (u/ml)	Relative activity(%)
5.8	0.27	6.2	0	0
6.2	8.03	8.1	7.29	92.7
6.6	7.96	8.4	6.96	88.5
7.0	7.94	8.5	7.86	100
7.4	8.07	8.3	5.57	70.9
7.8	8.08	8.3	5.86	74.9

(a) See the text for the experimental conditions.

Table 5. Effect of aeration on enzyme yield.(a)

Culture volume(m*l*)	DCW (g/liter)	Final *p*H	Activity (unit/ml)	Specific activity (unit/mg DCW)
25	6.63	8.90	13.7	20.7
50	7.45	8.81	14.06	1.89
100	6.57	8.82	4.09	0.62

(a) See the text for the experimental conditions.

and casamino acidgave relatively high enzyme activities. This observation seems to suggest that there might be a certain substance in the casein hydrolyzates that stimulates or influences the production of benzyl penicillin amidase Moreover, when NH_4Cl and yeast extract was used as nitrogen source, the enzyme production was fairly good in spite of the poor growth. The exact mechanisms and interactions related to these interesting observations would require more of refined experiments.

5. **Effect of indtcer on enzyme production.** Phenylacetic and benzoic acids have been used as inducers for benzyl penicillin

amidase biosynthesis] by many workers [6,8,11,31~34]. As shown in Figs. 7 and 8, phenylacetic acid showed maximal induction effect at a concentration of 0.15%, whereas benzoic acd, 0.1%. These inducers did not affect the cell growth significantly. Maximal activity attained by addition of benzoic acid (0.1%) was higher by a few % than that obtained by PAA (0.15%). Thus, it may be concluded that both inducers are about equivalent although there might be slight economic advantage of using benzoic acid over the PAA due to slightly lower cost of benzoic acid.

6. Effect of the initial *p*H of medium on enzyme production. As shown in Table 4, when the initial *p*H of medium was 5.8, there was neither the cell growth nor the enzyme activity. Within the range of *p*H 6.2 to 7.8, there was fairly good enzyme activity and growth. In the range of *p*H 6.2 to 7.0, relatively high level of enzyme activity was observed, whereas, in the range of *p*H 7.4 to 7.8, slight decrease in enzyme activities was observed. In all subsequent fermentation experiments the medium was adjusted to *p*H 7.0.

7. Effect of aeration on enzyme production. Fermentations were carried out with various culture volume to examine the aeration effect on enzyme production (Table 5). When the culture volume was 25ml or 50ml, the enzyme activity was about 3 times higher than 100m*l*. Thus, it may be concluded that highly aerobic condition is responsible for the good production of benzyl penicillin amidase from our mutant strain of *B. megaterium*. The oxygen transfer rate determined by the sulfite oxidation method using 25m*l* water in 500ml flask under the given operating conditions corresponding to the enyme production was about 56 m*M* of O_2/liter/hour.

8. Conclusion. Based on our experimental studies with the mutant strain of *Bacillus megaterium*, the optimum composition of the medium found was: casitone (Difco), 2.5%; yeast extract, 0.5%; glucose, 1.0%; benzoic acid, 0.1% or phenylacetic acid, 0.15%; and silicone antifoam, 0.01%. The optimal operating conditions of fermentation for enzyme production were; initial *p*H of medium, 7.0; temperature of cultivation, 28 to 30°C; oxygen transfer rate, greater than 55 m*M* of oxygen per liter per hour: and culure peried, 48—72 hours.

Under these optimal environmental conditions of fermentation, the benzyl penicillin amidase activity attainable was about 14 units per ml of fermentation broth.

A modified assay method for benzyl penicillin amidase that has a significant advantage over the conventional hydroxylamine assay method, has been developed and employed in this research.

摘 要

Bacillus megaterium ATCC 14945의 變異株로부터 penicillin amidase를 생산하는데 관여하는 여러가지 培養環境因子와 該酵素定量法의 새로운 變法을 검토하였다.

1. 효소 생산의 최적 배지 조성은 casitone 2.5%, yeast extract 0.5%, glucose 1%, potassium benzoic acid 0.1%(„ potassium phenylacetic acid 0.15%), silicone antifoam 0.01%이었다.

2. 上記 배지 조성하에서 初期 培地의 *p*H; 7.0. 溫度 培養; 28~30°C, oxygentransfer rate; 55m *M* of O_2 per liter per hour 이상으로 48—72 시간배양하였을 때 14 units per m*l* of broth의 효소역가가 기측되었다.

3. 該酵素의 定量法으로 사용되어 온 hydroxyl amine 法(유기용매 추출단계 介在)을 염산 첨가 前處理를 행함으로써 추출단계 없이 간편하게 定量' 가능하게 되었다.

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