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Production of Hydantoinase from *Streptomyces* sp.

Kwon, Tae-Jong, Joo-Kyung Lee* and Sang-Hoon Lee[1]

Department of Microbiological Engineering, College of Engineering, Kon Kuk University
[1]Department of Bioengineering, Central Research Laboratories of YungJin Pharmaceutical Co., Ltd.

방선균으로부터 Hydantoinase의 생산

권태종 · 이주경* · 이상훈[1]

건국대학교 공과대학 미생물공학과

[1]영진약품공업 (주)중앙연구소 생물공학연구부

In order to investigate hydantoinase-producing strain of the genus *Streptomyces*, 523 strains of *Streptomyces* sp. isolated from soils were cultivated in various media and conversion activity of the enzyme was measured to DL-5-phenylhydantoin. A number of strains producing hydantoinase were detected and among them, the strain of *Streptomyces* sp. Y-183 was selected as a most powerful strain to producing the enzyme. The optimal culture conditions for the production of hydantoinase of the strain were studied, and it was found that almost all hydantoinase activity was produced in the cell fraction. The maximum activity of the enzyme, 17.8 units/g of dried cells weight, was obtained when the strain was cultured at 30°C for 72 hr in a medium containing 1.0% of glycerol, 0.5% of yeast extract, 0.5% of soytone, 0.5% of beef extract, 0.6% of KCl, 0.002% of K_2HPO_4, 0.25% of $CaCO_3$, 0.0002% of $ZnSO_4$, 0.0002% of $FeSO_4$, and 0.4% of uracil as an inducer, and the pH of culture broth was adjusted ranging from 7.0 to 7.5.

Several D-amino acids, for example, D-*p*-hydroxyphenylglycine, D-phenylglycine and their derivatives are known to be important components of semisynthetic penicillin or cephalosporin antibiotics (1-3).

As an amino acid nonexisting in nature, D-*p*-hydroxyphenylglycine, and D-phenylglycine are chemically synthesized as racemic mixture and next separated into enantiomers by chemical or enzymatic methods. Chemical methods employed selected chiral agents for isolation of optically pure D-*p*-hydroxyphenylglycine or D-phenylglycine (4-8).

Hitherto, several enzymatic methods have been reported for the conversion of DL-5-substituted hydantoins into N-carbamyl-amino acids and to D-amino acids (9-12).

It was found that 5-substituted hydantoins were hydrolyzed by hydantoinase isolated from *Pseudomonas striata* and *Agrobacterium radiobacter* (13, 14).

Recently, it has been reported by Yamada *et al.* (13) on *Streptomyces almquisti* ATCC 618 and *Streptomyces griseus* ATCC 10137. With the purpose of producing hydantoinase by enzymatic conversion of DL-5-substituted hydantoins, the authors searched for microorganism producing higher hydantoinase activity, which stereospecifically hydrolysis DL-5-substituted hydantoins to yield N-carbamyl amino acids and now found a strain *Streptomyces* sp. Y-183 from soil.

In this report, the authors wish to described the screening method of the strain and optimal culture conditions of the strain to produce hydantoinase.

Key words: *Streptomyces* Hydantoinase
*Corresponding author

Materials and Methods

Chemicals

5-substituted hydantoins were prepared from the corresponding amino acids according to the procedure of Suzuki *et al.* (15).

N-carbamyl-amino acids were prepared by the reaction of amino acids with potassium cyanate (16).

DL-amino acids were purchased from Sigma chemical company. All other culture media were obtained from Difco laboratories and the other chemicals were commercial products of analytical grade.

Screening method of microbe capable of producing hydantoinase

The independent isolates designated *Streptomyces* sp. were isolated from soils by the isolation methods of International Streptomyces Project (I.S.P.) (17).

The first screening for hydantoinase-producing strains was performed as follows.

Spores of each strain grown for 1 to 2 weeks on I.S.P. No.5 agar slant medium were inoculated to 20 m*l* of medium containing 1.5% of glucose, 0.5% of yeast extract, 0.5% of soytone, 0.5% of beef extract, 0.6% of KCl, 0.25% of $CaCO_3$, 0.002% of K_2HPO_4, 0.0002% of $FeSO_4$, 0.0002% of $ZnSO_4$ and 0.3% of DL-5-(2-methylthioethyl)-hydantoin as an inducer, culture broth pH was adjusted to 7.0.

The cultivation was carried out at 30°C in 125 m*l* flasks with a rotary incubator shaker (G-25R, N. B.S., U.S.A.) at 220 rpm.

After the cultivation was continued for 48 hr and then the grown cells were separated from the culture broth by centrifuge and washed with saline. The collected cells were suspended in 20 m*l* of 100 mg of DL-5-phenylhydantoin dissolved in 0.02 M of potassium phosphate buffer (pH 8.0) was added to the cell of each strain, while each supernatant solution was mixed with an equal volume of 100 mg of DL-5-phenylhydantoin dissolved in same buffer.

These suspension and mixed solution were reacted for 40 hr at 30°C by water bath shaker (G-76D, N.B.S., U.S.A.) with gentle shaking.

After reaction, cells in the suspensions were removed by centrifugation for 10 min at 8000 × g and the supernatant solutions were analyzed by thin-layer chromatography.

While, in the case of mixed solutions, the solutions were directly subjected to thin-layer chromatography.

Thin-layer chromatography was carried out on Kiesel Gel F_{254} (type 60, E. Merck, W. Germany) with the following solvents system; n-butanol: acetic acid: water (4:1:1).

After the chromatograms was sprayed with *p*-dimethylaminobenzaldehyde reagent and heated in an oven for 5 min at 100°C, the relative amounts of producing N-carbamyl-phenylglycine were read with TLC chromatoscanner (CS-930, Shimadzu, Japan) by comparison with the known amount of authentic N-carbamyl-phenylglycine.

The secondary screening was carried out by the following method. Strains which appeared to produce hydantoinase were cultivated again in 20 m*l* of the above mentioned aqueous medium in 125 m*l* flasks at 30°C for 48 hr on a rotary incubator shaker operating at 220 rpm then the harvested cells were suspended in 20 m*l* of 0.02 M of potassium phosphate buffer (pH 8.0), disrupted by the ultrasonic cell disruptor (Virsonic, Virtis, U.S.A.) for 60 min at 20 KHz and at 4°C, centrifuged for 10 min at 12000 × g, and hydantoinase activities in the supernatant solution were determined.

Determination of hydantoinase

As almost all the hydantoinase activity produced by *Streptomyces* sp. was found in the cell fraction, the enzyme activity in the extract from the cells was determined as follows. The harvested cells from 20 m*l* of culture broth were suspended in 20 m*l* of 0.02 M of potassium phosphate buffer (pH 8.0), treated in an ultrasonic cell disruptor for 60 min at 4°C and separated off the cell debris by centrifugation for 10 min at 12000 × g.

100 mg of DL-5-phenylhydantoin as a substrate was add to the above supernatant solution and make up a total volume of 20 m*l* with above same buffer and reacted for 40 hr at 30°C with gentle shaking.

Quantitative determination of N-carbamyl-phenylglycine concentration was carried out colorimetrically according to the method of Cecere *et al.* (9) as follows.

A two milliliter of the reaction mixture was taken and the reaction was terminated by the addition of 0.5 m*l* of 12% (w/v) trichloroacetic acid, following 0.5 m*l* of 10% (w/v) *p*-dimethylaminobenzaldehyde in 6 N-HCl. Then, the mixture was diluted with 3.0

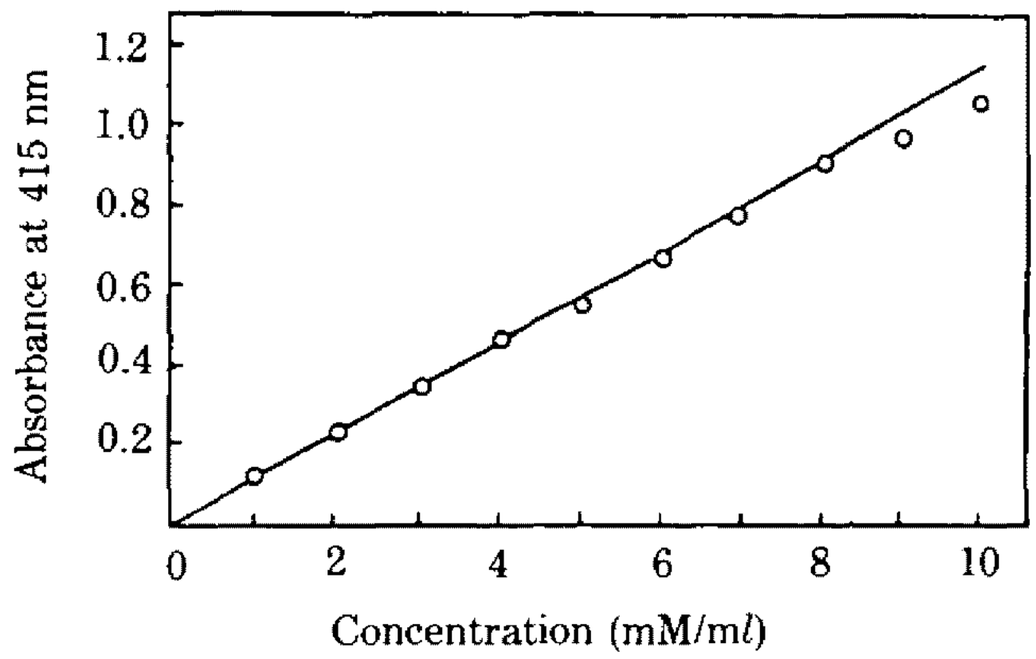

Fig. 1. Standard graphs determined on UV-Spectrophotometer with reaction mixture.

ml of water. After the precipitates of above mixture were removed by centrifugation, the absorbance at 415 nm was determined by UV-spectrophotometer (UV-240, Shimadzu, Japan).

The enzyme activity was determined by measuring the amount of N-carbamyl-phenylglycine formed from the DL-5-phenylhydantoin. As shown in Fig. 1, the absorbance was proportional to the N-carbamyl-phenylglycine concentration up to about 8 mM.

One unit of hydantoinase activity was defined as the amount of enzyme required to produce 1 μmol of N-carbamyl-phenylglycine per minute under assay conditions.

Culture conditions of microbes

For determining the optimal culture medium, spores of the strain were inoculated to 20 ml of the aqueous medium mentioned in the foregoing paragraph contained in 125 ml of flasks for 120 hr at 30°C with rotary incubator shaker at 220 rpm.

For studies on growth kinetics, change of the pH and accumulation of the enzyme, 20 ml of samples were taken during the course of the cultivation at various times to obtain data for enzyme formation and the samples were kept in refrigerated until analyzed. All of the media contained 0.6% of KCl, 0.002% of K_2HPO_4 as mineral sources, 0.0002% of $FeSO_4$, 0.0002% of $ZnSO_4$ as trace elements and 0.3% of DL-5-(2-methylthioethyl)-hydantoin as an inducer of the enzyme production. The cultivated cells were harvested by centrifugation and washed with saline and then the dried cells weight recorded after lyophilized by freeze dryer (Virtis, U.S.A.) at -60°C for 24 hr.

Enzyme preparation

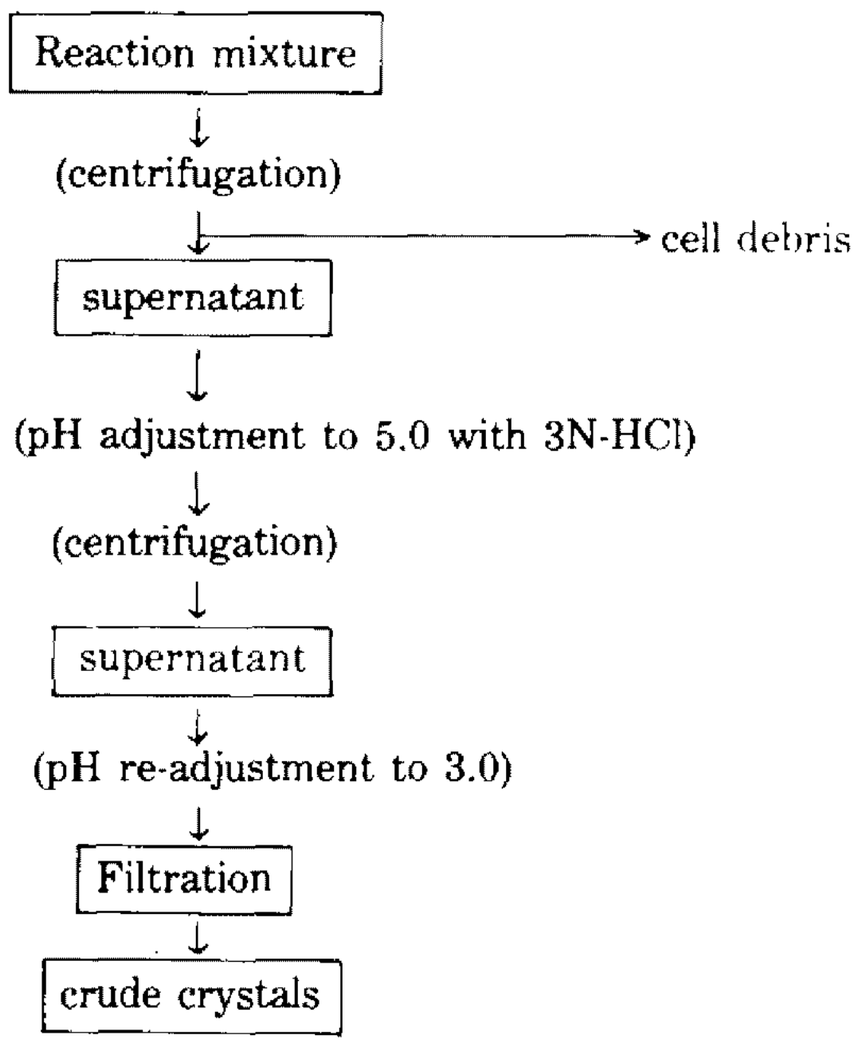

Fig. 2. Isolation procedure for N-carbamyl-amino acids from the reaction mixture.

The harvested cells obtained from 100 ml of culture broth were suspended in 100 ml of 0.5 mM of potassium phosphate buffer (pH 8.0) and disrupted by ultrasonic cell disruptor for 60 min at 4°C.

The resulting slurry was centrifuged at 12000 $\times$ g for 10 min to separate off the cell debris.

The clear supernatant was collected and maintained at 4°C until used as an enzyme source.

Analytical method

For isolation and identification of the products, 200 ml of the reaction mixture was reacted at 30°C for 40 hr with the cells harvested from 200 ml of culture broth.

The identities of isolated N-carbamyl amino acids and DL-5-substituted hydantoins were evaluated according to the melting point, I.R. spectra and the specific optical rotation by Polarimeter (DIP-360, JASCO, Japan). The isolation method of N-carbamyl amino acid was summarized in Fig. 2.

Results

Distribution of hydantoinase activity in *Streptomyces* sp.

Five hundred and twenty three species of *Streptomyces* were submitted to the screening test and 16 strains were selected as the hydantoinase-producer. Among them, higher activity was detected in strain

Table 1. Comparison of hydantoinase activity of *Streptomyces* sp.

Strains	Dry cell weight (mg/ml)	enzyme activity (unit/ml)	specific activity (unit/mg)
Y-4-1*	4.5	3.8	0.84
100	4.2	3.4	0.81
183	4.9	5.1	1.04
232	4.8	4.6	0.96
398	4.2	3.3	0.79
478	4.2	3.8	0.90
*Streptomyces*** *griseus* ATCC 10137	4.3	3.6	0.84

Composition of media were as follows;
*: 1.5% of glucose, 0.5% of yeast extract, 0.5% of beef extract, 0.5% of soytone, 0.6% of KCl, 0.25% of $CaCO_3$, 0.002% of K_2HPO_4, 0.0002% of $FeSO_4$, 0.0002% of $ZnSO_4$, 0.3% of DL-5-(2-methylthioethyl)-hydantoin and the pH was adjusted to 7.0.
**: 2.0% of glucose, 0.1% of beef extract, 0.25% of yeast extract, 1.0% of soybean meal, 0.5% of $(NH_4)_2SO_4$, 0.4% of KCl, 0.4% of $CaCO_3$, 0.02% of K_2HPO_4, 0.3% of DL-5-(2-methylthioethyl)-hydantoin and the pH was adjusted to 7.0.

Y-4-1, 100, 183, 232, 398, 478 and these six strains produced a large amounts of hydantoinase.

In all cases, the hydantoinase by these selected strains were intracellular enzyme.

As shown in Table 1, *Streptomyces* sp. Y-183 produced the most active hydantoinase activity than the other selected strains and this was adopted as a strain for further experiments to establish the most suitable conditions for the production of hydantoinase.

Effect of carbon sources on the formation of hydantoinase

Among glucose and five sugars tested, poly or oligosaccharides such as soluble starch, maltose, galactose and sucrose were unfavourable to the growth of cells and enzyme activity as shown in Table 2. Whereas, glucose and glycerol were favourable. Glycerol was the most suitable carbon sources as shown in Table 2.

The optimal concentration of glycerol was found to be 10.0 mg/ml of culture broth.

Effect of nitrogen sources on the formation of hydantoinase

Basal medium was used after supplementation with various organic and inorganic nitrogen sources at 1.0%, 1.5% and 2.0% respectively.

As shown in Table 3, addition of combined mixture of 0.5% of yeast extract, 0.5% of soytone and 0.5% of beef extract to the medium was more favourable for the enzyme production than organic or inorganic nitrogen source used only.

Table 2. Effect of carbon sources on enzyme formation.

Carbon	(mg/ml)	Dry cell weight (mg/ml)	enzyme activity (unit/ml)	specific activity (unit/mg)	final pH
Glycerol	10	5.0	6.21	1.24	6.69
	20	5.1	6.18	1.21	6.86
	30	5.2	6.14	1.18	6.94
Glucose	10	4.8	4.95	1.03	6.61
	20	4.9	5.20	1.06	6.70
	30	5.0	5.46	1.09	6.71
Sucrose	10	3.7	1.96	0.53	6.41
	20	3.8	1.97	0.52	6.40
	30	3.8	1.96	0.52	6.38
Maltose	10	4.1	2.73	0.67	6.53
	20	4.1	2.74	0.67	6.52
	30	4.2	2.80	0.67	6.53
Soluble starch	10	3.7	2.37	0.64	6.89
	20	3.8	2.39	0.63	6.90
	30	3.8	2.37	0.62	6.91
Galactose	10	3.8	2.12	0.56	6.88
	20	3.8	2.14	0.56	6.90
	30	3.8	2.14	0.56	6.92
None		3.4	2.32	0.68	6.87

Basal medium contained 0.5% of yeast extract, 0.5% of beef extract, 0.5% of soytone, 0.6% of KCl, 0.25% of $CaCO_3$, 0.002% of K_2HPO_4, 0.0002% of $FeSO_4$, 0.0002% of $ZnSO_4$, 0.3% of DL-5-(2-methylthioethyl)-hydantoin and the pH was adjusted to 7.0.
The enzyme activity was determined by measuring the amount of N-carbamyl-phenylglycine formed in 20 ml of reaction mixture described in the "methods" for the studies of the culture conditions. Specific activity was expressed as units per mg of dried cells.

Table 3. Effect of nitrogen sources on enzyme formation.

Nitrogen sources	(mg/m*l*)	Dry cell weight (mg/m*l*)	Enzyme activity (unit/m*l*)	Specific activity (unit/mg)
	10	4.8	4.58	0.96
Yeast extract	15	5.0	4.53	0.91
	20	5.1	4.41	0.86
	10	3.9	3.20	0.82
Soytone	15	4.0	3.40	0.85
	20	4.5	3.80	0.84
	10	4.6	4.15	0.90
Beef extract	15	5.0	4.10	0.82
	20	5.0	4.10	0.82
	10	3.2	0.70	0.22
Soybean oil	15	3.2	0.72	0.23
	20	3.3	0.72	0.22
	10	3.4	1.95	0.57
Tryptone	15	3.4	1.90	0.56
	20	3.3	1.86	0.56
	10	3.7	2.71	0.73
Casitone	15	4.0	2.71	0.68
	20	4.1	2.80	0.68
	10	3.5	1.62	0.46
Peptone	15	3.7	1.66	0.45
	20	3.7	1.68	0.45
	10	4.0	2.51	0.62
$NaNO_3$	15	4.3	2.46	0.57
	20	4.3	2.40	0.56
	10	4.3	2.47	0.57
NH_4Cl	15	4.0	2.31	0.58
	20	3.8	2.10	0.55
	10	4.5	3.70	0.82
$(NH_4)_2SO_4$	15	4.7	3.60	0.77
	20	4.7	3.60	0.76
control		5.3	6.43	1.21

Effect of hydantoin derivatives on the formation of hydantoinase

Table 4. Effect of hydantoin derivatives on the formation of hydantoinase.

Hydantoin derivatives (3.0 mg/m*l*)	Dry cell weight (mg/m*l*)	Enzyme activity (unit/m*l*)	Specific activity (unit/mg)
Hydantoin	5.75	3.54	0.62
DL-5-methylhydantoin	5.41	2.43	0.45
DL-5-(2-methylthioethyl)-hydantoin	5.31	6.44	1.21
DL-5-phenylhydantoin	5.34	3.33	0.62
DL-5-*p*-hydroxyphenyl hydantoin	5.52	3.61	0.65
DL-5-isobutylhydantoin	4.31	2.08	0.48
DL-5-indolymethyl hydantoin	4.33	2.81	0.65
DL-5-isopropylhydantoin	3.89	1.42	0.37
Allantoin	5.93	1.83	0.31
Uracil	5.43	7.82	1.44
Dihydrouracil	5.47	7.84	1.43
None	5.30	1.26	0.24

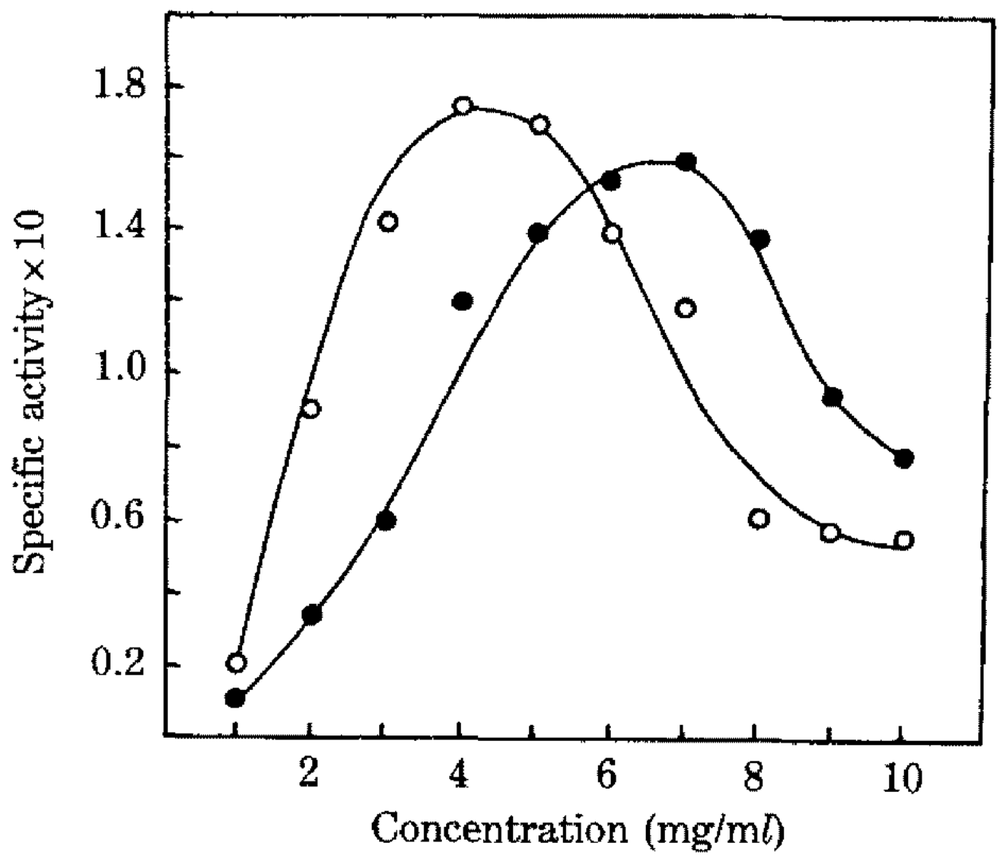

Fig. 3. Effect of inducer concentration on hydantoinase production.
○; Uracil ●; Dihydrouracil

The microorganism was cultivated in basal medium supplemented with 3.0 mg/m*l* of various hydantoin derivatives.

As shown in Table 4, the highest activity was induced by uracil and dihydrouracil.

As shown in Fig. 3, the optimal concentration of uracil and dihydrouracil was found to be 4.0 mg/m*l*

Table 5. Effect of initial pH on the formation of hydantoinase.

Initial pH	Final pH	Dry cell weight (mg/ml)	Enzyme activity (unit/ml)	Specific activity (unit/mg)
5.0	6.18	3.59	2.51	0.70
5.5	6.42	4.70	5.00	1.06
6.0	6.72	4.82	6.12	1.27
6.5	6.88	4.96	6.61	1.33
7.0	6.95	5.30	8.82	1.66
7.5	7.04	5.38	9.56	1.78
8.0	7.34	5.38	8.68	1.61
8.5	7.64	5.01	6.24	1.25
9.0	7.89	4.74	5.06	1.07
9.5	8.42	3.27	2.24	0.69

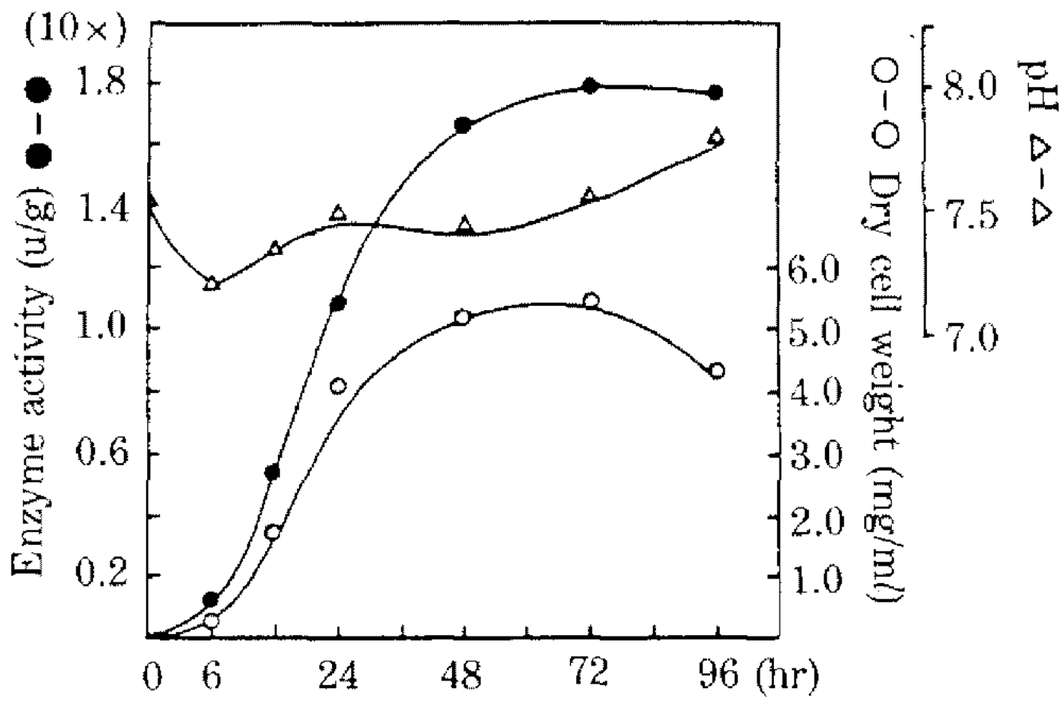

Fig. 4. Time-course on the formation of hydantoinase.

and 7.0 mg/ml respectively.

Effect of initial pH on the formation of hydantoinase

The effect of initial pH of the culture medium on the formation of hydantoinase was investigated by using the optimal hydantoinase media of which the pH was varied with 1 N-HCl or 1 N-NaOH.

As shown in Table 5, a desirable initial pH was found to be between 7.0 and 7.5.

The lower or higher initial pH of media probably caused the delay of growth and subsequently the reduction of enzyme formation.

The highest activity was obtained with the media of pH 7.5.

Table 6. Properties of isolated N-carbamyl-amino acids.

N-carbamyl-amino acid	Melting point (°C)	Specific optical rotation $[\alpha]_D^{20}$
N-carbamyl-methionine*	162 – 163	+23.7
Authentic sample	161 – 162	+24.0
N-carbamyl-phenylglycine**	200 – 202	−134
Authentic sample	193 – 200	−136

*: C = 1 in 1N-HCl, **: C = 1 in 0.1N-NH_4OH

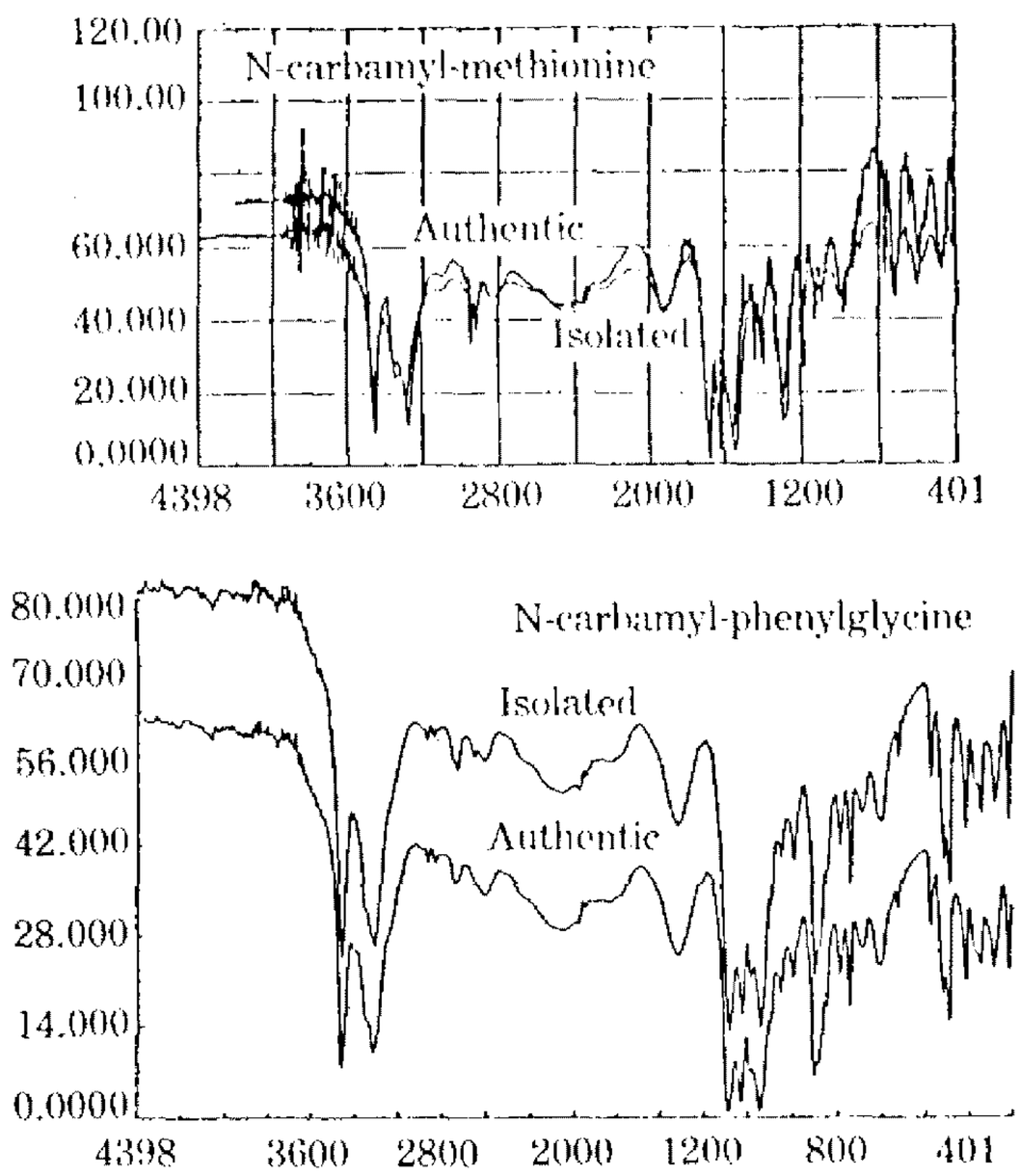

Fig. 5. I.R. Spectrum of isolated N-carbamyl-amino acids.

Effect of time-course on the formation of hydantoinase

Fig. 4 shows a typical time-course of growth and enzyme formation in its optimal medium. After a long lag period, cell growth and enzyme accumulation started. Hydantoinase formation was found to be parallel with the cell mass increase in the logarithmic growth phase. The maximum enzyme activity reached after 72 hr cultivation and it attained to 17.8 unit per gram of dry cell weight.

Enzymatic hydrolysis of DL-5-substituted hydantoins

요 약

Hydantoinase (EC 3.5.2.2) 생산을 위해 토양에서 분리한 균주 Y-183을 배양시킨 결과 Glycerol이 탄소원으로 양호했으며, 질소원으로는 혼합된 형태의 질소원이 요구되었다. 특히 Yeast ext.와 Beef ext.가 효소생성에 크게 영향을 미쳤고, Hydantoin 유도체 및 Pyrimidine 유도체의 영향을 조사한 결과는 Uracil이 가장 양호한 결과를 나타냈다. 배양시간은 방선균의 특성상 72시간의 배양이 필요했으며 효소의 생성은 24시간 이후에 왕성하였다. 생성된 Hydantoinase는 균체내에 효소를 축적했으며 이들을 추출하여 기질로서 DL-5-Phenylhydantion 등과 반응시킨 결과 N-carbamyl-D-amino acids가 생산되는 것이 분석결과 확인되었다.

An enzyme that obtained according to enzyme preparation method, were added as an enzyme source to reaction mixture containing of DL-5-(2-methylthioethyl) hydantoin and DL-5-phenylhydantoin as substrate. After reaction with the determination method of hydantoinase, each product was isolated by the procedure shown in Fig. 2.

The infra-red spectra of each of the isolates were shown in Fig. 5 that were in good agreement with the authentic sample.

These isolate also showed almost the same specific optical rotation value as those of authentic sample as shown in Table 6 and were in good agreement in the literature data (18-20).

Discussion

Hydantoinase has already been found in *Actinomycetaceae* such as *Streptomyces* (11), nevertheless there have been hardly any reports of hydantoinase with *Streptomyces*.

In this paper, we described the screening of hydantoinase-producing *Streptomyces* and screened several *Streptomyces* strains which can produced N-carbamyl-amino acids from DL-5-substituted hydantoins from natural sources.

Among them, *Streptomyces* sp. Y-183, isolated from soil sample, showed the highest activity when the DL-5-phenylhydantoin was used as a substrate instead of DL-5-(2-methylthioethyl) hydantoin (11).

This phenomenon was very different from that in the case of *Pseudomonas striata* (11) and the amounts of hydantoinase produced by the *Pseudomonas striata* was reported to be 1 to 2 units per m*l* of culture broth.

The steric configuration of the end product was found to the D-form of N-carbamyl-amino acids.

According to these results, this strain was selected for the studies of culture conditions.

Our results on optimization of the culture conditions were observed that the mixture of yeast extract, soytone, and beef extract is the best nitrogen source and also found the maximum level of enzyme activity which could be increased further by the addition of uracil as an inducer.

Selected enzyme from this work will now be purified and more fully characterized.

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