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Inhibitory Substance Produced by *Aspergillus* sp. on the Snake Proteinase — Culture Conditions for the Production of Inhibitor —

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Aspergillus 屬 菌株가 生成하는 蛇毒 proteinase에 對한 沮害物質
— 沮害物質의 生産條件 —

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***Aspergillus* sp. MK-24 was cultured at 30°C in the medium consisting of 2% glucose, 0.2% $NaNO_3$, 0.02% K_2HPO_4, 0.02% $MgSO_4$ $7H_2O$, 0.02% KCl, and at initial pH of 5.0. The production of the inhibitor on venom proteinase reached to the maximum in 7 days. Sodium nitrate or potassium nitrate as a nitrogen source was favorable. The production of inhibitor was not affected by the addition of most of the inorganic salt used but depressed by lead, zinc, cobalt, mercuric or silver salts.**

The venomous snakes are classified according to morphological characteristics and comprise five families (1,2); *Crotalidae, Viperidae, Elapidae, Hydrophiidae* and *Colubridae*. Three Korean poisonous snakes *(Agkistrodon bromohoffi brevicaudus, Agkistrodon caliginosus, Agkistrodon saxatilis)* belongs to the *Crotalidae* familiy (3-5). In pathogenesis of Korean poisonous snake *Agkistrodon* families, the biological properties of venom proteinase have not yet been studied although some toxic factors related to hemorrhang and edema (7) were separated from the *A. bromohoffi brevicaudus* venom.

Snake venoms are recognized as proteinous substance to have enzymatic actions. For inactivation of snake venoms, chemical methods (8-11) are generally applied. All inhibitory effect of these chemicals is due to nonspecific inhibitory action.

An inhibitor produced by *Aspergillus* sp. MK-24 was obtained and the purification, stabilities and biological activities of the inhibitor were reported in the previous paper (12). The culture conditions for the production of inhibitor are described in this paper.

Key words: Snake proteinase, inhibitor production
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Materials and Methods

Microorganism

The microorganism used in this experiment was *Aspergillus* sp. MK-24, which was isolated from soil and was maintained on potato agar.

Cultivation methods

A 100 ml-Erlenmeyer flask containing 20 ml of basal medium was inoculated with 0.1 ml of spore suspension of *Aspergillus* sp. MK-24 and cultivated at 30°C for 7 days with standing culture.

The basal medium (pH 5.0) was composed of 2.0% glucose, 0.3% $NaNO_3$, 0.02% KCl, 0.02% K_2HPO_4, 0.02%$MgSO_4 \cdot 7H_2O$. The role of amino

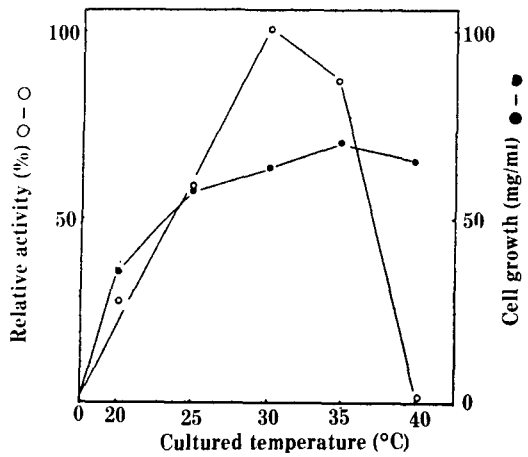

Fig. 1. Effect of temperature on production of the inhibitor and cell growth.

acids for the inhibitor production was determined with a concentration of 0.05% in the basal medium. The effect of vitamins was studied with a concentration of 2.5 μg/ml in the basal medium.

Determination of the inhibitory activity

The proteinase activity of snake venom was determined by the Folin-Ciocalteu method (13). The ratio of inhibition was estimated by the method described previously (12).

Estimation of cell growth

Cultured microorganisms were gathered by filtration and then washed with distilled water 3 times. Thereafter it was dried at 105 °C for overnight and weighed.

Results and Discussion

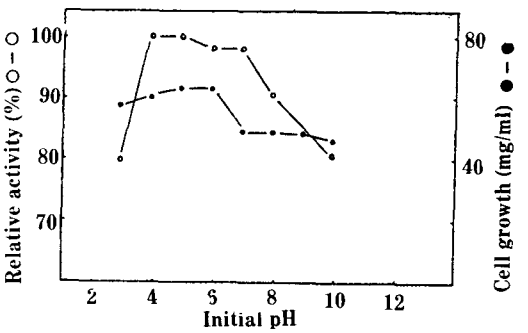

Fig. 2. Effect of initial pH on the production of the inhibitor.

Effect of temperature and pH

The temperature and pH for production of inhibitor in the basal medium are illustrated in Fig. 1 and 2, respectively.

As shown in Fig. 1, the maximal inhibitor was attained at 35 °C. Fig. 2 shows the effect of initial pH of the medium on growth and production of the inhibitor, and pH 5 was revealed most favorable for the cell growth and the inhibitor production.

Sources of carbon

The effect of different carbons on the inhibitor production was studied. From the results shown in Table 1, it was found that among the carbon sources used except arabitol, xylitol, salicin, was specifically favorable. So we chose glucose as carbon source and examined optimal concentration for the production of inhibitor. The concentration

Table 1. Effect of carbon source on the inhibitor production

Carbon source	Growth	Final pH	Relative activity
D-Glucose	+ + +	5.0	100
D-galactose	+ +	6.6	85
D-Mannose	+ + +	5.6	95
D-Sorbitol	+ + +	6.0	92
D-Fructose	+ + +	4.4	100
D-Arabinose	+	5.4	88
D-Arabitol	+	5.6	—
D-Ribose	+ + +	5.0	95
D-Rhamnose	+ +	6.2	94
L-Sorbitol	+ + +	6.4	97
D-Xylose	+ + + +	7.2	90
Mannitol	+	5.6	62
I-Inositol	+ + +	7.2	38
Xylitol	+	5.8	—
Glycerine	+ + +	6.8	90
Maltose	+ + +	5.0	94
Lactose	+	5.8	85
Raffinose	+ + +	7.0	90
Inulin	+ + +	5.6	94
Dextrin	+ + +	6.2	82
S-Starch	+ + + +	6.8	96
Salicin	+	6.0	—
Sucrose	+ + +	4.8	100

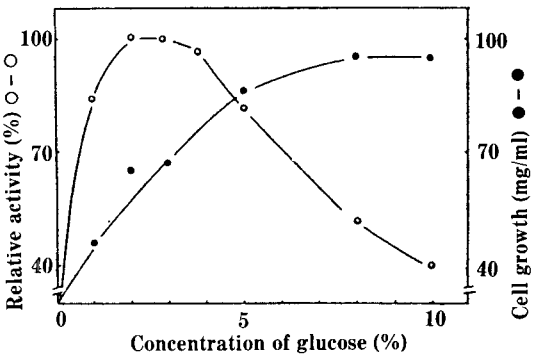

Fig. 3. Effect of concentration of glucose on the inhibitor production.

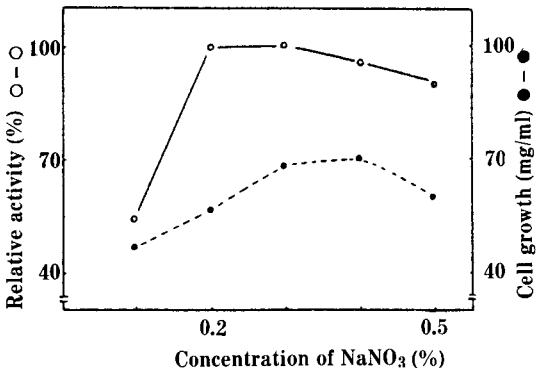

Fig. 4. Effect of sodium nitrate as a accessory nitrogen source on production of the inhibitor.

of glucose in the medium affected either cell growth or the inhibitor production (Fig. 3). The optimal concentration of glucose for the production of inhibitor was about 2%, but 8% glucose was suitable for the cell growth.

Sources of nitrogen

The effect of nitrogen sources was tested by adding several kinds of inorganic nitrogen sources. When organic nitrogen sources were used, cell growth was better than inorganic nitrogen sources, but the production of the inhibitor was less than inorganic nitrogen sources. As shown in Table 2, sodium nitrate was proved to be a best suitable nitrogen source for the inhibitor production. The optimal concentration of sodium nitrate was 0.2-0.3 %, but the best cell growth was occurred at 0.4% (Fig. 4).

Table 2. Effect of nitrogen source on the inhibitor production

Nitrogen source	Growth	Final pH	Relative activity
(NH_2)	+ + +	6.5	82
$(NH_4)_2SO_4$	+ +	3.6	21
$(NH_4)_2HPO_4$	+ + +	3.6	24
NH_4NO_3	+ +	5.0	75
NH_4Cl	+ +	3.4	81
$NaNO_2$	+ +	5.8	51
$NaNO_3$	+ + +	5.8	100
KNO_3	+ +	6.4	90

Table 3. Effect of amino acid on the inhibitor production

Amino acid	Growth	Final pH	Relative activity
None	+ +	not	100
Leucine	+ + + +	specific	99
Threonine	+ + +	(5.4-5.8)	93
Isoleucine	+ +		108
Glutamic acid	+ + +		107
Methionine	+ + + +		97
Asparagine	+ + +		98
Histidine	+ +		93
Alanine	+ + +		98
Peptone	+ + +		105
Aspartic acid	+ +		109
Serine	+ + +		106
Proline	+ + +		94
Hydroxyproline	+ + +		94
β-alanine	+ +		105
Cysteine	+ +		103
Valine	+ +		103
Glutamine	+ +		97
Arginine	+ +		103
Ornithine	+ + +		89
Phenylalanine	+ +		98
Tryptophan	+ +		100
Glycine	+ +		94
Lysine	+ + +		88
Cystine	+ + + +		83
Tyrosine	+ + + +		95

Each amino acid was added with concentration of 0.05% to the basal medium.

Table 4. Effect of vitamin on the inhibitor production

Vitamin	Growth	Final pH	Relative activity
None	+ +	not	100
Biotin	+ + +	specific	97
Ca-Pantothenate	+ +	(5.0-5.4)	97
Thiamine	+ +		90
Ascorbic acid	+ + +		103
Pyridoxin	+ +		93
Inositol	+ +		95
Folic acid	+ + +		102
PABA	+ +		92
Nicotinic acid	+ + +		92
Riboflavin	+ + +		102
B_{12}	+ + +		101

Each vitamin was added with concentration of 25 μg/ml to the medium for the inhibitor production.

Table 5. Effect of metal salt on the inhibitor production

Metal salt	Growth	Final pH	Relative activity
None	+ +	not	100
$MgSO_4 \cdot 7H_2O$	+ + +	specific	98
$FeSO_4$	+ +	(5.0-5.4)	97
$CoCl_2$	+		4
$ZnCl_2$	+ + + +		21
$AgNO_3$	+		4
$CuSO_4$	+		66
$HgCl_2$	-		—
$Pb(NO_3)_2$	+		50
$MnCl_2$	+ +		95
$MgCl_2$	+ +		92
$CaCl_2$	+ +		99

Each metal salt was added with concentration of 1 ml to the medium containing 2% glucose, 0.3% $NaNO_3$, 0.02% K_2HPO_4, and 0.02% KCl.

Effect of amino acid and vitamin

The effect of various amino acids for the production of inhibitor to the basal medium was investigated. Among the amino acid used, lysine and cystine repressed slightly on the inhibitor production (Table 3). All the vitamins used did not affect on the production of inhibitor and cell growth (Table 4).

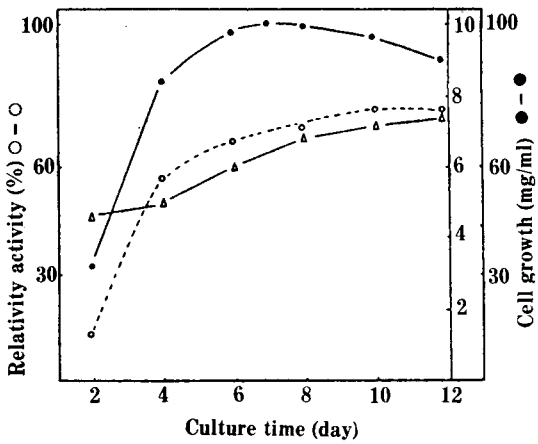

Fig. 5. Time course of inhibitor production

Effect of metal salts

$HgCl_2$, $AgNO_3$, $CoCl_2$, $ZnCl_2$, and $Pb(NO_3)_2$ repressed severely the cell growth and the inhibitor production, but another metal salts used did affect on the production of the inhibitor (Table 5).

Cultivation time course

For the production of the inhibitor, cell growth and variation of pH was determined with the elapse of cultivation time. Cultivation was carried out at 30°C with 200 ml of medium in 1000 ml Erlenmeyer flask by the standing. As shown in Fig. 5, the inhibitor was produced maximally in 7 days of cultivation.

요 약

Aspergillus 屬 菌株 MK-24로부터 venom proteinase inhibitor의 生産條件을 검토한 결과 질소원으로서는 有機能窒素가 菌生育에 있어서는 매우 좋았으나 物質生成에 있어서는 無機能窒素보다 못하였다. 無機窒素源으로서는 sodium nitrate가 가장 좋은 效果를 보였다.

炭素源으로서는 glucose가 大部分 糖類와 비슷한 結果를 나타내었고 특히 arabitol, xylitol, salicin 등은 本 菌株가 炭素源으로 利用치 못하는 것으로 추정되었으며, vitamin 類는 物質生成에 무관하였으며 金屬鹽類로서는 效果的인 것은 없으나, Ag^+, Co^{++}, Zn^{++} 等은 억제하였다.

培養温度와 pH는 각각 30℃와 pH 5가 菌의 生育과 沮害物質生成에 제일 적당하였고, 炭素源과 窒素

源으로서 glucose 2%, $NaNo_3$ 0.3%을 加한 液体培地에서 7 日間 정치배양하였을 때 沮害物質生育이 가장 좋았다.

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