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Porduction and Enzymatic Characteristics of α-Galactosidase from *Aspergillus niger*

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Aspergillus niger 에 의한 α-Galactosidase 의 생산 및 효소적 특성

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Abstract

α-Galactosidase from *Aspergillus niger* as a possible enzyme for removal of flatulence factors in soybean foods was produced the highest in 120 hours in either Czapeck-Dox liquid medium or wheat bran solid medium. The most efficient carbon and nitrogen sources in Czapeck-Dox medium were raffinose and sodium nitrate, respectively, whereas the addition of the sources showed negative effects in wheat bran. pH optima for enzyme activity and stability were 4.0-5.0 and 3.5-6.5, respectively, and optimum temperature for stability was 40-50℃. Upon reaction on p-nitrophenyl-α-D-galactoside, Michaelis constant was 0.42 mM and maximum velocity was 152 μmoles substrate/minute/kg solid medium. Mercuric chloride acted as a strong noncompetitive inhibitor and p-chloromercuribenzoate, even in low concentration, acted as a competitive inhibitor. Crude α-galactosidase hydrolyzed raffinose and stachyose completely, giving spots of monosaccharides only on thin-layer chromatogram.

Introduction

Soybean contains several hazardous constituents such as trypsin inhibitor, hemagglutinin, phytate and flatulence factor. For the removal of flatulence factors including raffinose and stachose, several methods such as soaking[1], germination[2], ultrafiltration[3] and enzymic method have been reported. The production of α-galactosidase useful in the enzymic method was attempted from plants[4] as well as microorganisms[5-10] in foreign countries. However, any effort to produce α-galactosidase from fungi is very limited in Korea[11,12].

This study was, therefore, carried out to examine the production of α-galactosidase from *Aspergillus niger* and its enzymatic characteristics for the removal of flatulence factors in soybean foods.

Materials and Methods

Microorganism and cultivation

All experiments were made with *Aspergillus niger* NRRL 337, maintained in the Department of Biology, Ewha Woman's University. The organism on a slant culture was inoculated to 20 ml of Czapeck-Dox medium in a 100 ml Erlenmeyer flask and grown on a reciprocal shaker (5cm amplitude of vibration, 100 rpm) for 24 hours. Then 0.1 ml of the culture was used to inoculate 50ml of Czapeck-Dox medium in a 250 ml Erlenmeyer flask and cultivated on a reciprocal shaker for 5 days at 30℃.

To examine a possibility of industrial application, 8 ml of water was added to 10 g of wheat bran in a 300 ml Erlenmeyer flask and sterilized. The bran medium was inoculated with 1 week -old organisms and incubated for 5 days at 30℃ as a stationary culture.

Preparation of crude enzyme

Liquid cultures were homogenized for 10 min and separated by filtration to obtain a crude enzyme solution.

To the wheat bran culture was added 60 ml of distilled water, homogenized for 10 min after one hour and then filtered. The residue was washed with 40 ml of distilled water and the washing was combined with the filtrate.

Assay of α-glactosidase

As a standard method, the reaction mixture containing 2 ml of 2 mM p-nitrophenyl-α-D -galactoside (PNPG) in 0.05M McIlvaine buffer, pH 6.5 and 1 ml of enzyme solution was incubated at 40℃ for 10 min. The reaction was terminated by adding 5 ml of 1M Na_2CO_3. The amount of released p-nitrophenol was determined by means of Spectronic 21 spectrophotometer at 400nm.

One unit of α-galactosidase was defined as the amount of enzyme which release 1 μmole of p -nitrophenol per minute under the above conditions.

Michaelis constant (Km) and maximum velocity (Vmax)

With various concentrations of PNPG, enzyme reaction was run for 10 and 20 min. The result of enzyme assay was used to calculate the initial velocity by extrapolation. Michaelis constant and maximum velocity were graphically determined by the method of Lineweaver-Burk's double reciprocal plot[13].

Measurement of hydrolysis rate

The amount of p-nitrophenol was estimated to follow the degree of PNPG hydrolysis by crude enzyme. The amount of reducing sugar liberated during hydrolysis of the natural substrate, raffinose, was measured by Somogyi's iodometry[14] to follow the degree of enzymatic hydrolysis. Reducing sugar was expressed as galactose equivalent. When 1 ml of 3 mM raffinose was fully hydrolyzed by 1 ml of crude enzyme solution, the amount of produced reducting sugar would be 1.62 mg (180.16×0.003×3)

A reaction mixture containing 1ml of 3mM sucrose and 1ml of crude enzyme was also incubated to find out the presence of sucrase activity. Provided that sucrose was hydrolyzed at 100%, the amount of reducing sugar produced would be 1.08 mg (180.16×0.003×2).

Thin-layer chromatography (TLC)

Qualitative identifications of sugars in enzyme digests were made by thin-layer chromatography as described earlier[12].

Results and Discussion

Conditions for enzyme production

1) Incubation time and pH

As shown in Fig. 1, α-galactosidase production by *Asp. niger* reached the maximum in 120 hours at 30℃ in both Czapeck-Dox liquid medium and wheat bran solid culture.

The effect of pH on the enzyme production in Czapeck-Dox liquid medium is shown in Fig. 2. The optimum pH for its production was pH 6.

2) Carbon and nitrogen sources

The effects of various carbon sources on the enzyme production are shown in Table 1. In Czapeck-Dox liquid medium, addition of any carbon source increased the production of enzyme. Especially, the addition of raffinose, arabinose, sucrose and melibiose greatly increased α-galactosidase production. On the other hand, the addition of any other carbon source to wheat bran solid culture rather lowered the

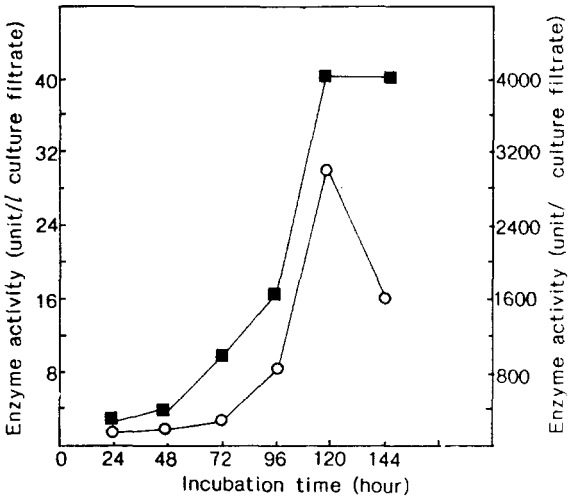

Fig. 1. Time course of α-galactosidase production from *Asp. niger*
■——■; wheat bran solid medium
○——○; Czapeck-Dox liquid medium

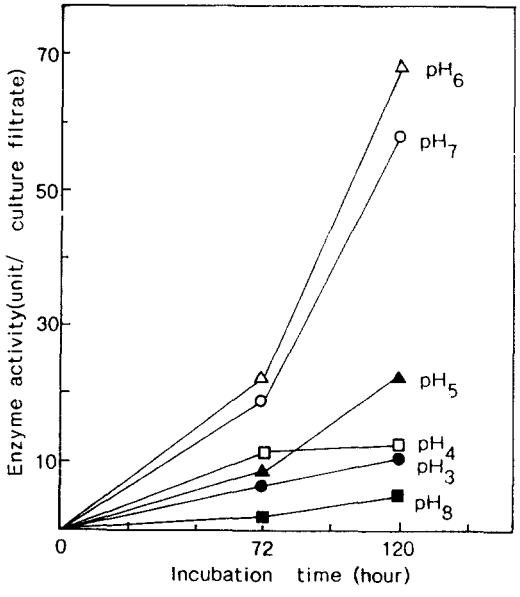

Fig. 2. Effect of pH on α-galactosidase production from *Asp. niger* in liquid culture
pH 3-6; 0.1M McIlvaine buffer
pH 7-8; 0.1M Tris-HCl buffer

enzyme production since the natural carbon source in the medium was presumably sufficient as energy source and inducer whereas the added

Table 1. Effect of carbon source on the α-galactosidase production from *Asp. niger*

Nitrogen source added*	Relative activity (%)	
	Czapeck-Dox medium	Wheat bran
Control (none)	100	100
Arabinose	770	41
Fructose	409	46
Galactose	174	57
Galactouronic acid	9	52
Glucose	234	33
Lactose	134	39
Melibiose	687	41
Pectin	341	41
Raffinose	800	69
Sorbose	7	41
Sucrose	751	43

* The level of carbon source was 5% in Czapeck-Dox medium and 2% in wheat bran. The organism was grown for 5 days at 30℃ by shaking culture in liquid medium and by stationary culture in solid medium

Table 2. Effect of the level of carbon source on the α-galactosidase production in Czapeck-Dox liquid medium*

Sucrose (%)	Relative activity (%)
none	100
1	461
3	498
5	751
8	410

* The organism was grown for 5 days at 30℃ by shaking culture

sources acted as energy source only. As shown in Table 2, the optimum level of sucrose in Czapeck-Dox medium was 5%.

The effects of nitrogen sources on the enzyme production are given in Table 3. Addition of $NaNO_3$ or NH_4NO_3 to Czapeck-Dox liquid medium tended to increase the enzyme production whereas no effect was observed in wheat bran solid medium.

Table 3. Effect of nitrogen source on the α-galactosidase production from *Asp. niger*

Carbon source added*	Relative activity (%)	
	Czapeck-Dox medium	Wheat bran
Control (none)	100	100
$NaNO_3$	1152	94
NH_4Cl	36	73
$(NH_4)_2SO_4$	172	87
$(NH_4)_2HPO_4$	288	90
NH_4NO_3	352	94
Casein	140	90
Peptone	216	79
Soybean extract	124	73
Urea	6	82

* The level of nitrogen source was 1% in Czapeck-Dox medium and 0.5% in wheat bran. The organism was grown for 5 days at 30℃ by shaking culture in liquid medium and by stationary culture in solid medium

Enzymatic characteristics

1) Optimum pH temperature

As shown in Fig. 3. The optimum pH for the enzyme activity was pH 4.0-5.0, which was similar to that reported for a commercial enzyme from *Asp. niger.*[6].

As indicated in Fig. 4, crude enzyme was relatively stable between pH 4 and 6 under the conditions tested. At the pH value 6.5 of natural soymilk, the enzyme activity was not lost appreciably.

The effect of temperature on the enzyme stability is shown in Fig. 5. The maximum activiity was observed in the range of 40-50℃. Above this temperature, the activity declined very rapidly and was almost negligible above 60℃. Accordingly, the crude enzyme shoud be used below 45℃ in applications.

2) Effect of substrate concentration

The effect of substrate concentration on enzyme activity was examined by incubating reaction mixtures containing 5 different concentrations of PNPG for 10 min and 20 min at 40℃. A Lineweaver-Burk plot made it possible to obtain Km value of 0.42 mM and Vmax value of 152 μmoles substrate/min/kg solid medium for PNPG as shown in Fig. 6. From these values, the

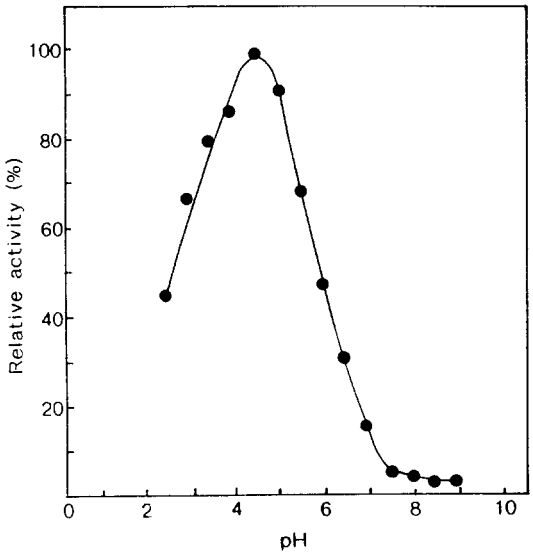

Fig. 3. pH-activity curve of crude α-galactosidase from *Asp. niger*

pH 2.5 - 7.5; McIlvaine buffer
pH 7.5 - 9.0; Tris-HCl buffer

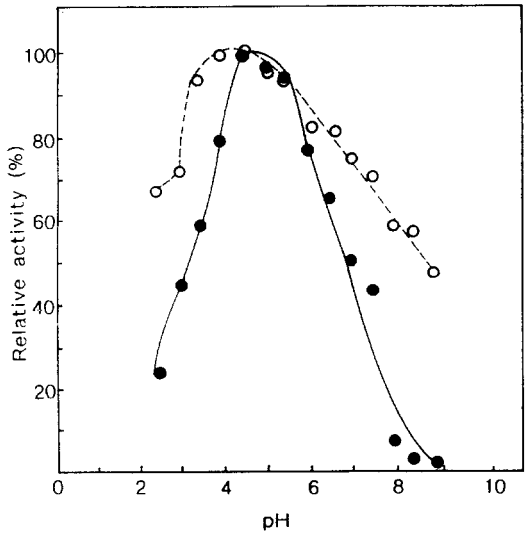

Fig. 4. pH - stability curve of crude α-galactosidase from *Asp. niger*

Enzyme solution in 0.01M buffer was preincubated at 55℃ for 20 min (●——●) or at 5℃ for 96 hours (○——○) and its actiivity assayed at pH 6.5

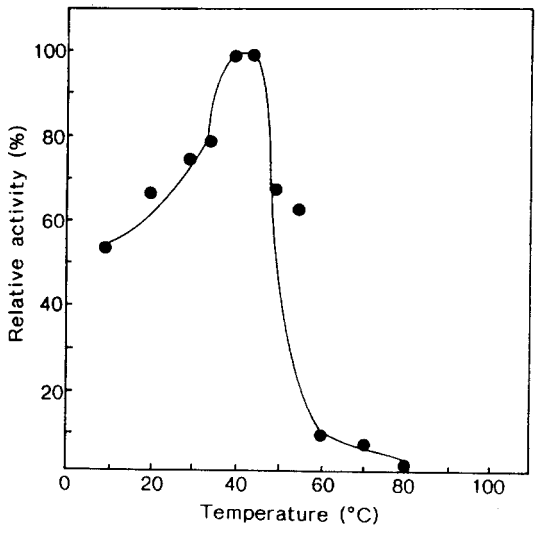

Fig. 5. Temperature-stability curve of crude α-galactosidase from *Asp. niger*. Enzyme solution was preincubated at the indicated tem perature for 15 min and then the residual activity determined at 40℃

Table 4. Effect of inhibitors on the α-galactosidase activity from *Asp. niger*

Inhibitors	Final concentration(M)	Relative activity(%)
None		100.0
p-Chloromercuribenzoate	2×10^{-5}	28.4
Cysteine	1×10^{-3}	30.0
EDTA	1×10^{-3}	114.5
8-Hydroxyquinoline	1×10^{-3}	90.5
Iodine	5×10^{-4}	88.5
KCN	1×10^{-3}	90.5
Sodium pyrophosphate	4×10^{-2}	31.1
Metal ions		
$AgNO_3$	1×10^{-3}	42.2
$CaCl_2$	1×10^{-3}	89.6
$CuCl_2$	1×10^{-3}	88.6
$FeCl_3$	1×10^{-3}	107.0
$HgCl_2$	1×10^{-3}	14.1
KCl	1×10^{-3}	89.6
$MgCl_2$	1×10^{-3}	89.3
$Pb(NO_3)_2$	1×10^{-3}	102.7
$ZnCl_2$	1×10^{-3}	85.3
Sugars and organic acid		
D-Fructose	1×10^{-2}	30.8
D-Galactose	1×10^{-2}	56.4
D-Glucose	1×10^{-2}	56.4
Sodium citrate	1×10^{-2}	61.6
Sucrose	$1\times10_{-2}$	41.1

affinity of the enzyme for PNPG appears to be relatively high in comparison with affinity for natural substrates.

3) Effect of inhibitors

α-Galactosidase activity was assayed in reaction mixture containing specified concentration of inhibitors to investigate the effects of inhibitors on the enzyme activity. The results are summerized in Table 4. Among the organic compounds tested, p-chloromercuribenzoate (PCMB) was found to cause an inhibition at the lowest concentration. This result suggested that α-glactosidase requires sulfhydryl groups for its activity similar to the enzyme from *Aspergillus saitoi*.[5] However, it appeared to be different from α-galactosidase of *Calvatia cyathiformis*[7] which was not inhibited by sulfhydryl blocking agents.

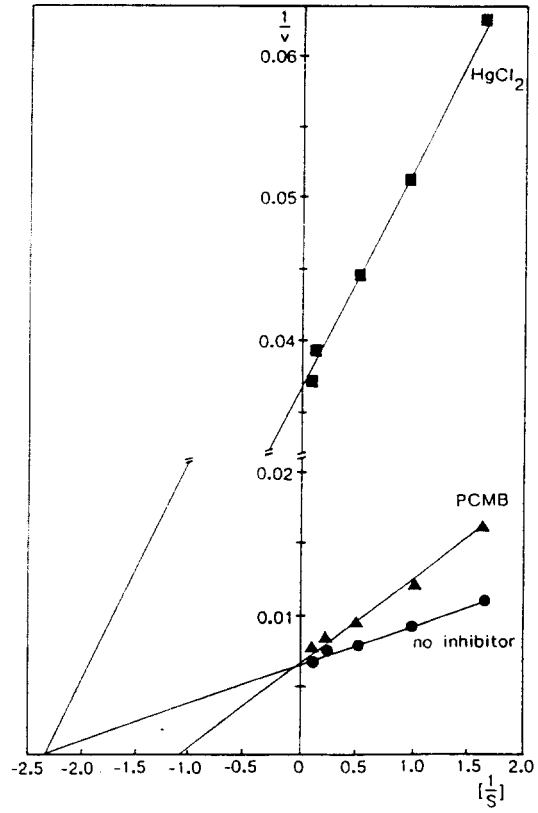

Fig. 6. Lineweaver-Burk plots of crude α-galactosidase in the absence or presence of PCMB or $HgCl_2$

As shown in Fig. 6, PCBM acted as a competitive inhibitor and it seems that para-substituted phenyl groups of PCMB and PNPG compete for the binding site of the enzyme. Among the inorganic compounds, $HgCl_2$ had a strong inhibitory effect as reported for commercial enzyme from *Asp. niger*[6] and α-galactosidase from *Asp. saitoi*[5]. As indicated in Fig. 6, $HgCl_2$ acted as a noncompetitive inhibitor, probably causing denaturation of the enzyme protein as reported for α-galactosidase from *Calvatia cyathiformis*. $AgNO_3$ caused a slight inhibition. The inhibition of all sugars appeared to be non-specific.

Enzymatic hydrolysis of substrates

The enzyme from *Asp. niger* hydrolyzed 95% of PNPG within 2 hours. The hydrolysis of raffinose as a natural substrate reached 65% within 3 hours. When sucrose was employed as a substrate in consideration of coexistence of sucrase activity in crude enzyme, it was completly hydrolyzed within 3 hours. The lower rate of hydrolysis in case of raffinose is likely due to the fact that sucrase can not act on raffinose unless galactose residue is first removed by α-galactosidase activity.

Sugar constituents of the enzymatic hydrolysates of raffinose and stachyose by the enzyme were identified by TLC and it was found that the crude enzyme hydrolyzed raffinose and stachyose completely, giving spots of monosaccharides only ultimately.

요 약

大豆중에 존재하는 腸內 가스 발생인자인 raffinose와 stachyose의 제거에 이용될 수 있는 *Aspergillus niger*의 α-galactosidase는 Czapeck-Dox 액체배지와 밀기울 배지에서 5일간 배양하였을 때 효소생성이 제일 높았다. Czapeck-Dox 액체배양에서 raffinose와 $NaNO_3$가 효소생산에 가장 효과적인 탄소원과 질소원으로 나타났으며 밀기울 배양에서는 질소원 또는 탄소원의 첨가효과가 없었다. 생성된 粗酵素용액의 작용 최적 pH는 4.0-5.0, 안정도 최적 pH는 3.5-6.5, 최적 온도는 40-50℃ 였다. 합성기질인 p-nitrophenyl-α-D-galactoside에 대한 Michaelis 상수는 0.42 mM, 최대반응속도는 152μmoles substrate/min/kg (고체배지)이었다. $HgCl_2$는 강력한 비경쟁 저해제로 작용하였고 p-chloromercuribenzoate는 낮은 농도에서 경쟁 저해제로 작용하였다. 조효소 용액은 raffinose와 stachyose를 완전 분해하여 단당류 만을 생성하였다.

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