SELECTION OF FUNGAL STRAINS WHICH PRODUCE THE ENZYMES ACTING ON ARABINAN AND SOME PROPERTIES OF THE ENZYMES

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Introduction

The enzyme hydrolyzing arabinan to L-arabinose was first demonstrated in Taka-diastase by EHRLICH *et al*[1, 2]. KAJI and co-workers[3~5] examined the activities produced by *Aspergilli* and *Clostridia*, and confirmed that these may be involved at least two different enzymes; one hydrolyzes arabinan exowisely to release L-arabinose only (exo-enzyme), and the other attacks a random position of the arabinan molecule to give L-arabinose and its oligosaccharides as the hydrolytic products (endo-enzyme). From the results surveyed on the enzyme productivity in various phytopathogenic fungi, FUCHS *et al*[6] recognized that arabinanases present in the culture filtrates are constitutive or inducible exo-enzyme. As reported previously[7, 8], an enzyme which was isolated from the culture filtrate of *Aspergillus niger* is α, L-arabinofuranosidase. Recently the author in collaboration with KAJI succeeded in crystallization of the α,L-arabinofuranosidase.

The present paper is concerned with the selection of arabinanase producers from filamentous fungi, and with some properties of the enzymes in order to prospect the existence of endo-enzymes in the fungal strains.

Materials and Methods

Organisms and culture: The fungal strains used in this experiment were kindly supplied from the Department of Fermentation Technology, Osaka University, and the laboratories of the Microbiological Chemistry and of the Phytopathology. These strains were maintained on the potato agar slant containing 2% D-glucose.

Each fungus was inoculated to a bran culture medium composed of 5 g of wheat bran and 8 ml of water in a 50 ml Erlenmeyer flask and cultured at 25°C for an appropriate period. The culture was added with 10 times its weight of water, and the enzymes were extracted for 5 hr at 10°C by occasional stirring. After filtration through cloth, the filtrate was centrifuged and dialyzed against tap water for 20 hr at 2°C. The clear dialyzate was used for the assay of arabinanase activity.

Measurement of activity: The reaction mixture composed of 3 ml of 1% aqueous solution of beet-arabinan, 1 ml of buffer and 1 ml of enzyme solution was incubated at settled temperatures. Except where stated otherwise, reaction was proceeded at 40°C and pH 4.0, using 0.1 M citric acid-0.2 M sodium monophosphate buffer. After 2 hr incubation, a portion of the reaction mixture was withdrawn and the reducing value was determined by the Somogyi-Nelson's method[9 10]. One activity unit has been defined as that amount of enzyme which will liberate 1 μ mole of arabinose from beet-arabinan per min at 40°C.

Paper chromatography of sugars: Paper chromatography of the enzymatic digest of beet-arabinan was carried out by the ascending method using one of the following solvent systems: ethyl acetate-pyridine-water (12 : 5 : 4); upper layer of butyl alcohol-acetic acid-water (5 : 1 : 4). Spots were developed with aniline hydrogen phthalate[11] or alkaline silver nitrate[12].

Beet-arabinan: This was prepared by the method of Hirst and Jones[13] from beet pulp, and on acid hydrolysis, it gave L-arabinose (67.7%), D-galactose (24.0%) and a small quantity of D-galacturonic acid.

Experimental Results

1. Selection of the Potent Arabinanase Producers

Two hundred and twenty five strains were tested for their abilities of arabinanase production on the bran culture medium. The distributions of the enzyme productivity in various genera are listed in Table 1. As seen in the table, the strains which show high enzyme productivity were observed frequently in *Aspergilli* and *Penicillia* but rarely in *Rhizopus* and *Mucor*. All phytopathogenic fungi showed considerable arabinanase productivity suggesting their possible roles in the process of infection, since the enzyme may facilitate the penetration of the fungus itself into the plant tissues by a hydrolytic cleavage of arabinan contained in the cell wall materials. Out of 43 strains which showed the activities above 2 units, three strains, *Aspergillus niger* No. 5195, *Aspergillus japonicus* and *Sclerotinia sclerotiorum*, were selected as the most potent enzyme producers.

Table 1. Distributions of arabinanase producers in various genera.

Genus	Number tested	Number of strains which showed activities*		
		above 2 units	1~2 units	below 1 unit
Aspergillus	153	35	52	66
Penillium	14	3	4	7
Rhizopus	16	0	3	13
Mucor	12	0	3	9
Monascus	2	0	0	2
Neurospora	2	0	0	2
Chaetomium	4	0	0	4
Phycomyces	2	0	0	2
Other saprophytic fungi	5	0	0	5
Cladosporium	3	0	2	1
Fusarium	3	1	2	0
Sclerotinia	3	2	1	0
Other phytopathogenic fungi	6	2	3	1
Total	225	43	70	112

* The assay method is given in the text. Activity was expressed as units per g of koji.

2. Comparison of the Selected Strains on Their Abilities of Arabinanase Production

Experiments were conducted to decide the optimal conditions for the enzyme formation by the bran cultures of the selected strains. Fig. 1. shows the time courses of the enzyme formation when the fungi, *A. niger*, *A. japonicus* and *S. sclerotiorum*, were grown at the temperatures indicated.

For the cultures of two *Aspergilli* the optimal temperature appeared to be at 30°C, where the enzyme activities virtually reached the maximum level in 4 days, while it was found to be adequate at 25°C for the culture of *S. sclerotiorum*, and the maximum enzyme production was attained after 10 days.

The effects of defatted soybean and moisture content of the medium on the enzyme formation were also examined. In these experiments a part of wheat bran was substituted with the equivalent amount of defatted soybean and the moisture content was adjusted with

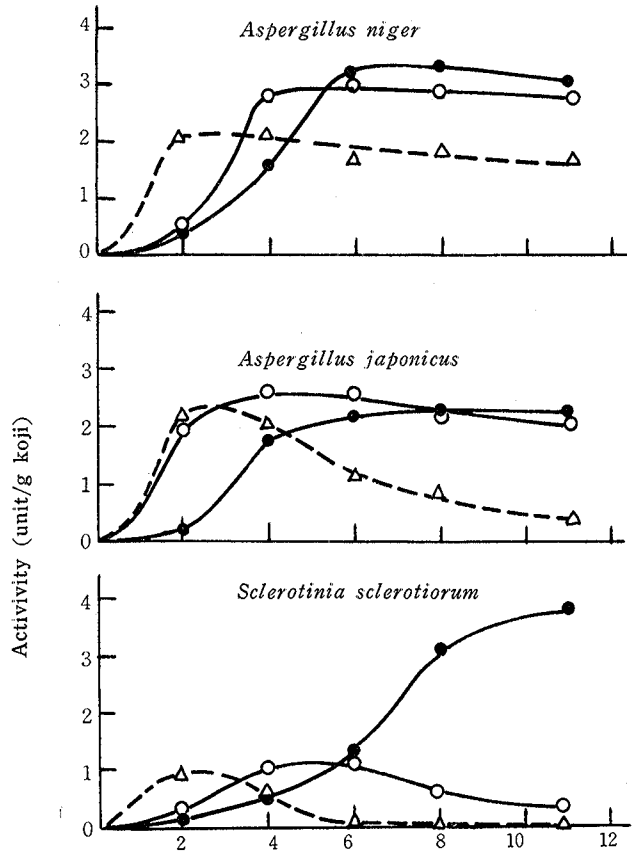

Fig. 1. Effect of temperature on the production of arabinanase by the selected strains.
Each strain was cultured on a medium composed of 20 g of wheat bran and the same weight of water in a 300 ml Erlenmeyer flask, at 25°(●——●), 30°(○——○) and 35°C (△———△), respectively.

Table 2. Effects of defatted soybean and moisture contents of the medium on arabinanase production by the selected strains.
A. niger and *A. japonicus* were cultured for 4 days at 30°C, and *S. sclerotiorum* was cultured for 10 days at 25°C. Other experimental details are described in the text.

Strain	Defatted soybean content (%)*	Moisture content (%)**	Arabinanase activity (unit/g of koji)
Aspergillus niger	0	100	3.06
	0	160	3.40
	25	100	5.07
	25	160	4.95
	50	120	4.70
	50	140	5.63
	75	120	4.86
	75	140	4.93
Aspergillus japonicus	0	120	2.82
	25	140	3.93
	50	100	4.70
	75	160	4.32
Sclerotinia sclerotiorum	0	140	3.63
	25	120	6.15
	50	140	6.20
	75	100	5.50

* Ratio of defatted soybean to the total solid materials;
** Ratio of water added to the total solid materials.

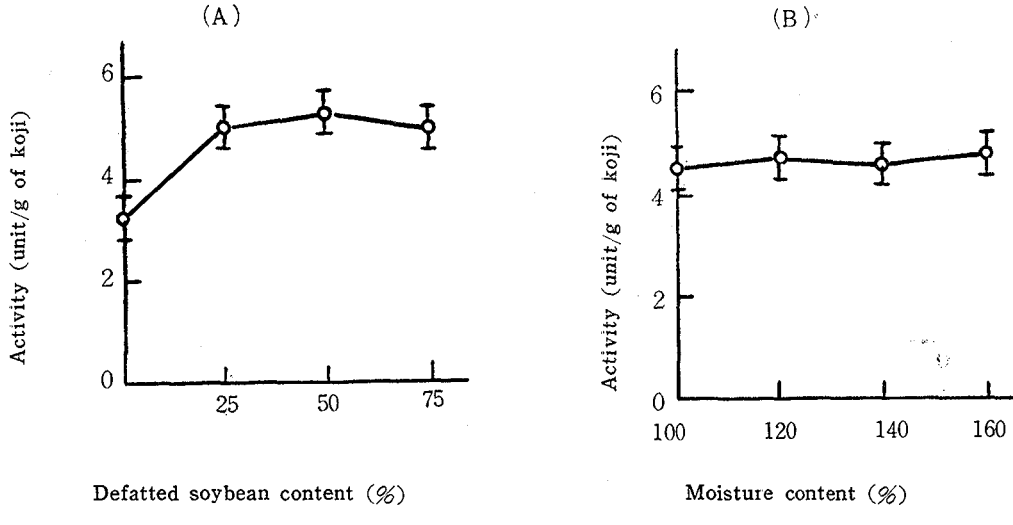

Fig. 2. Correlation between defatted soybean content and the enzyme production (A), and that of moisture content and the enzyme production (B). Each value was calculated from the datum shown in Table 2. The verticals indicate ± standard deviation.

a quantity of water added.

As shown in Table 2 and Fig. 2, the addition of defatted soybean affected considerably on the enzyme formation in all the cultures, showing much more enzyme production in the ranges of 25 to 50 % of defatted soybean contents, whereas the moisture content had little effect on the enzyme formation within the level tested. Since effectiveness of defatted soybean could not be substituted by the use of any other protein such as casein and peptone, it was reasonably considered that L-arabinose-containing polysaccharides in defatted soybean[14, 15, 16] may serve as inducer for the enzyme formation. Among these strains, S. *sclerotiorum* showed the highest arabinanase productivity but its culture required a long period. Therefore, *A niger* may be suitable for the large scale culture.

3. Some Properties of the Enzymes from the Selected Strains

The enzyme solutions used in these experiments were prepared as follows. The extracts of bran cultures prepared as mentioned above were fractionated with ammonium sulfate between 0.3 and 0.8 saturation, and the precipitated enzymes were dissolved in water and dialyzed against tap water for 24 hr at 2°C.

a) pH optima for the enzyme activities

The enzyme activities were measured at various pH ranges from 1.5 to 8.0 using the buffer systems: 0.1 M glycine-0.1 M HCl, below pH 2.5; 0.1 M citric acid-0.2 M Na_2HPO_4, between pH 2.5 and 7.0; 0.1 M HCl-0.1 M tris, above pH 7.0. As shown in Fig. 3, the pH optima appeared between 3.8 and 4.0 with the enzyme from *A. niger*, 4.0 and 4.5 with *A. japonicus* and 3.2 and 3.8 with *S. sclerotiorum*, respectively.

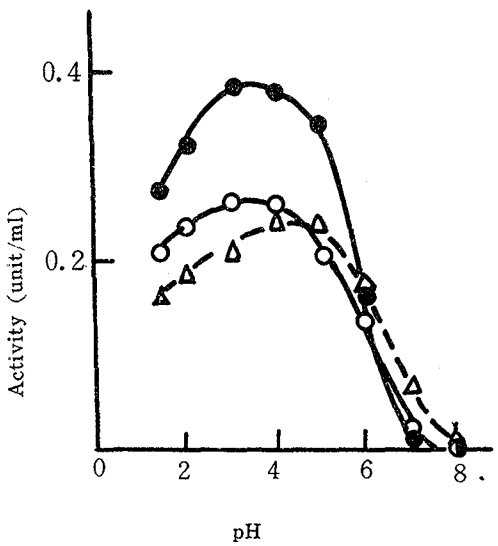

Fig. 3. Effect of pH on the arabinanase activities.
The three enzyme preparations obtained from *A. niger* (○——○), *A. japonicus* (△— —△) and *S. sclerotiorum* (●——●) were assayed at 40°C.

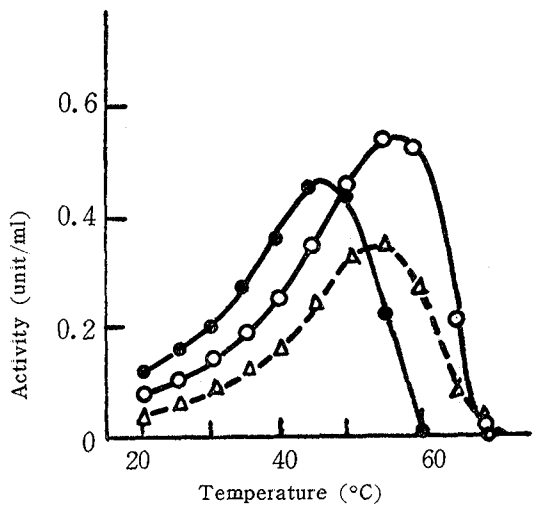

Fig. 4. Effect of incubation temperature on the arabinanase activities.
The enzymes from *A. niger* and *S. sclerotiorum* were incubated at pH 3.8 and that of *A. japonicus* at pH 4.2, respectively, for 2 hr. Symbols are the same as in Fig. 3.

b) Effect of temperature on the enzyme activities

The influence of reaction temperature on the enzyme activities was investigated in the regions of optimal pH for the respective enzymes.

As shown in Fig. 4, the maximum activities were found at 55°C with the enzymes of two *Aspergilli* and at 45°C with that of *S. sclerotiorum*, respectively. It is obvious that the temperature optimal for the enzyme activity correlates with that adequate for the enzyme production (refer to Fig. 1).

c) Effects of pH and temperature on the enzyme stabilities

Thermal stability of the enzymes was measured by exposing the buffered enzyme solutions with various pH values in test tubes to the settled temperatures for 10 min. After cooling by placing the tubes in an ice bath, the residual activities were determined at optimal pH for each enzyme activity, where the activities of the enzyme solutions without treatment were assigned to be 100 %. Fig. 5 shows the residual activities as a function of pH. It is evident that each enzyme is most stable in the region of the optimal pH for its activity, except that in the case of the enzyme from *A. niger*, it is quite stable at other pH, around 7.0, than that of optimum activity. At these pH regions, however, the enzyme from *S. sclerotiorum* was completely inactivated after 10 min exposure at 65°C, and those from *A. niger* and *A. japonicus* at 70°C, respectively, whereas at pH 7.0 the enzyme from *A. niger* is unusually stable and little lose of the activity was observed after 10 min heating at 70°C. These findings on the thermal stability of arabinanase from *A. niger* should be noted in consistent with the previous observations with the enzyme from Sanactase[17], an enzyme preparation from *A. niger*, and with the purified enzyme of *A. niger*[7].

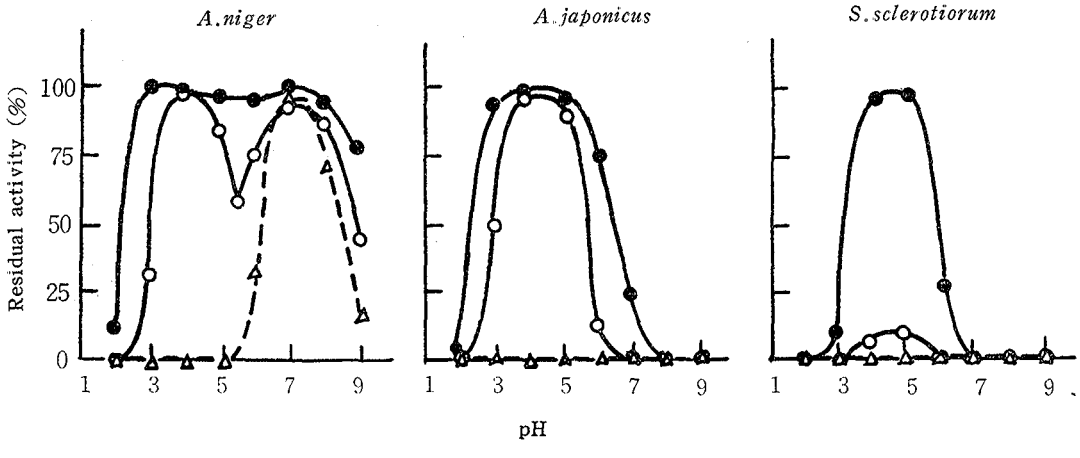

Fig. 5. Effects of pH and temperature on the enzyme stability.
The three enzyme preparations were buffered at various pHs and treated for 10 min at 50°(●——●), 60°(○——○) and 70°C (△— —△). The residual activities were assayed as described in the text.

d) Mode of action of the enzymes

To the reaction mixture multiplied to a total 15 ml was added a few drops of toluol to prevent microbial contamination, and the mixture was incubated at 30°C. At intervals, portions (1.0 ml) were removed and analyzed for reducing values increased by the Somogyi-Nelson's method and sugars liberated by paper chromatography, respectively. The progressive curves of beet-arabinan hydrolysis with the enzymes from three different strains are shown in Fig. 6, and paper chromatograms of the digests in Fig. 7.

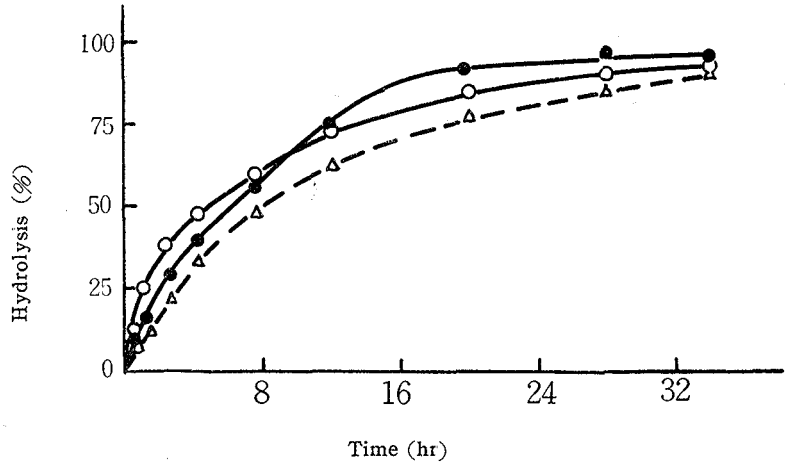

Fig. 6. Progressive curves of beet-arabinan hydrolysis.
Beet-arabinan (0.6% solution) was incubated with the same three enzyme preparations as in Fig. 3, at the optimal pH for respective enzyme activities and 30°C. Symbols are the same as in Fig. 3.

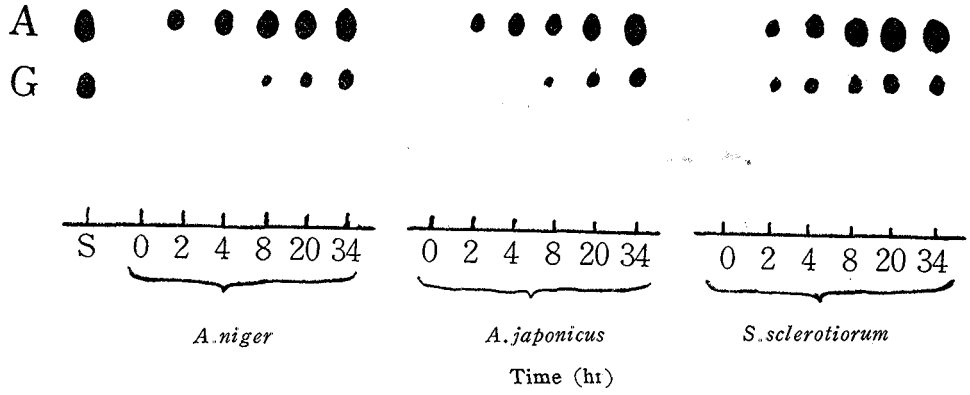

Fig. 7. Paper chromatograms of reducing sugars in the digests of beet-arabinan with the enzymes from strains indicated.
The digests, 10 μl, from the experiment shown in Fig. 6 were spotted on a Toyo filter paper No. 51A, and developed with ethyl acetate-pyridine-water (12 : 5 : 4).
S represents the standard sugar solutions. A and G indicate L-arabinose and D-galactose, respectively.

With each of the enzymes beet-arabinan was hydrolyzed almost completely, and monomer L-arabinose was always detected as the hydrolysis product, although D-galactose was also detected owing to hydrolysis of impure polysaccharides consisting presumably of D-galactose units[18, 19] by contaminated enzymes, because such a bran culture might contain many other enzymes capable of hydrolyzing various polysaccharides. In all reaction times, oligosaccharides of L-arabinose could not be detected, therefore it may be considered that the enzymes cleave L-arabinose units from the arabinan molecule predominately by the exo-wise fashion.

Discussion

The data so far available have indicated that the pH optima for arabinan hydrolysis with the enzymes from fungal strains lie in acidic regions ranging from 3.0 to 5.0. In the present investigation, similar results were obtained with the enzymes from *A. niger*, *A. japonicus* and from *S. sclerotiorum*; their pH optima proved to be 3.8 to 4.0, 4.0 to 4.5, and 3.2 to 3.8, respectively.

On the basis of results obtained in comparing the thermal stability of the enzymes from these strains, it is apparent that there is a close relationship between the stability of the enzymes themselves and the culture temperature adequate for the enzyme production. This is in agreement with the results obtained with enzymes from *Bacillus stearothermophilus* and *Bacillus cereus* by AMELUNXEN and LINS[20]. Moreover, the pH profile of thermal stability displays a close resemblance with the pH activity curve of the respective enzymes, except the case of the enzyme from *A. niger* in a neutral pH region. The enzyme from *A. niger* is stable at two pH regions of 4.0 and 7.0, suggesting a possible contribution of different enzymes.

However, in the experiments conducted with this enzyme so far, no separable activity could be detected, and therefore, further purification and characterization of the enzyme are required.

Each of the enzymes of these fungi hydrolyzed beet-arabinan almost completely to L-arabinose, but the hydrolysis patterns are not identical with each other. The rapid hydrolysis up to the extent of 30% is indicative that the enzymes preferably cleave one unit L-arabinose side chains, attached along the main L-arabinose chain of the arabinan molecule with α-(1-3) glycosidic linkages, since such linkages have been estimated to be about one third of the total in the arabinan molecule[21].

It is of particular interest to elucidate whether the enzyme hydrolyzing arabinan by a endowise fashion is existent in these fungal enzyme preparations.

Paper chromatograms of the digests did not reveal the presence of L-arabinose oligomers which may be characteristic products derived from the cleavage of arabinan by endo-enzyme. This result, however, cannot be followed directly by the concept that the endo-enzyme does not exist, as reported by FUCHS *et al*[6], because if the two enzymes, endo- and exo-enzymes, are concerned cooperatively with hydrolysis of arabinan, L-arabinose oligomers as the resulting products of the endo-enzyme action may be instantaneously subjected

to further hydrolysis by the action of exo-enzyme, so the monomer L-arabinose can be always detected in the digest. As judged from the hydrolytic data, the enzyme solution of *S. sclerotiorum* seems to be more contained with the endo-enzyme than those of the other two strains.

Summary

In order to investigate arabinanases from fungal strains, 225 strains belonging to various genera were tested for their abilities of the enzyme production on a bran culture medium. Three strains, *Aspergillus niger*, *Aspergillus japonicus* and *Sclerotinia sclerotiorum*, were selected as the most potent arabinanase producers. With these three strains, some culture conditions for the enzyme production were established; these proved to be adequate at 30°C for 4 days for the cultures of *A. niger* and *A. japonicus*, and at 25°C for 10 days for that of *S. sclerotiorum*.

Addition of defatted soybean (25 to 50% of the material) resulted in the effective enzyme production. All the enzymes from these strains hydrolyzed beet-arabinan almost completely to L-arabinose and the pH optima lay in 3.2 to 4.5. These enzymes are stable at optimal pH for the respective enzyme activities, but unlike the enzymes of the other strains, the enzyme from *A. niger* is unusually stable at pH 7.0, where it retained most of the activity after heating at 70°C for 10 min. The enzyme from *S. sclerotiorum* lost its activity completely when heated at 65°C for 10 min, and that of *A. japonicus* at 70°C for 10 min, respectively. The possibility of existence of the endo-enzyme which attacks the arabinan molecule randomly has been discussed.

Acknowledgment

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かびのアラバン分解酵素生産菌株の選択および酵素の若干の性質

田川　清

要　旨

かびの生産するアラバン分解酵素の一つは α, L-arabinofuranosidase であることは，既に梶と著者らの共同研究により明らかにされた。

アラバン分解酵素の優良生産菌株を得ること，および endo- 型酵素生産の可能性を求めて菌の選択を行ない，*Aspergillus niger* No. 5195, *Aspergillus japonicus*, *Sclerotinia sclerotiorum* の3株を選んだ。

液培養において，*A. niger*, *A. japonicus* は 30°C 4日間の培養で酵素生産が最高に達し，*S. sclerotiorum* は25°C 10日間で最高に達した。脱脂大豆の添加は酵素生産を増進させる。

これら3菌株の酵素はテンサイ・アラバンをほぼ完全にアラビノースに分解する，その作用最適 pH 域は 3.2～4.5 である。

酵素は作用最適 pH 域で安定であるが *S. sclerotiorum* の酵素は 65°C 10分，*A. japonicus* の酵素は 70°C 10分の加熱で完全に失活する。*A. niger* の酵素は中性附近の pH 域ですこぶる安定であり，70°C 10分の処理でほとんど失活しない。

アラバンの水解経過から endo-型 酵素存在の可能性について考察した。

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