Acta Pharmaceutica Turcica
XLII (2,3) (86-90) 2000

SOME EXTRACELLULAR GLYCOSIDASES OF ASPERGILLUS FLAVIPES

ASPERGILLUS FLAVIPES'IN BAZI EKSTRASELULER GLİKOZİDAZLARI

NURTEN ÖZSOY*, SEHER BİRTEKSÖZ, GÜLTEN ÖTÜK**, HAKAN BERKKAN***

Department of Biochemistry* and Pharmaceutical Microbiology**, Faculty of Pharmacy, University of Istanbul, 34452, Istanbul, Turkey

Aspergillus flavipes was studied for the production of some glycosidases. The highest yield of extracellular α- and β-glucosidases, α-galactosidase and α-amylase was obtained using maltose, cellobiose, melibiose and starch, respectively as a sole carbon source, with an incubation at 30°C for 11 days, 12 days, 13 days and 6 days, respectively. An initial pH of culture medium of 6 for α- and β-glucosidases; 5 for α-galactosidase; and 6.5 for α-amylase was found to be optimum. These conditions gave a yield of 9.1 U/ml for α-glucosidase, 112,5 U/ml for β-glucosidase, 88 U/ml for α-galactosidase and 3.5 U/ml for α-amylase. The results reported in this study indicate that Aspergillus flavipes is indeed an active source of several glycosidases.

Aspergillus flavipes'ten bazı ekstraseluler glikozidazların elde edilmeleri incelendi. En yüksek ekstraseluler α- ve β-glukozidaz, α-galaktozidaz ve α-amilaz verimi besiyerine sırasıyla maltoz, sellobioz, melibioz ve nişasta ilavesi ile elde edildi ve α- glukozidazın 11. günde, β-glukozidazın 12. günde, α-galaktozidazın 13. günde, α-amilazın 6. günde, 30°C'de sırasıyla 9.1 U/ml , 112,5 U/ml, 88 U/ml, 3.5 U/ml maksimum aktivite değerleri gösterdikleri saptandı. Besiyerlerinin optimum pH'larını saptamak amacıyla yapılan çalışmalarda α- ve β-glukozidaz pH 6, α-galaktozidaz pH 5, α-amilazın ise pH'sı 6.5 olan besiyerlerinde en yüksek aktivite gösterdikleri belirlendi. Sonuçlar, Aspergillus flavipes'in bazı glikozidazlar için zengin bir kaynak olduğunu göstermektedir.

Keywords: *Aspergillus flavipes*, extracellular glycosidases, culture conditions

Anahtar kelimeler: *Aspergillus flavipes*, ekstraseluler glikozidazlar, besiyeri koşulları

Introduction

Glycosidases, such as α- and β-glucosidases (EC 3.2.1.20 and EC 3.2.1.21), α- and β- galactosidases (EC 3.2.1.22 and EC 3.2.1.23) and α-amylase (EC 3.2.1.1) are important enzymes in scientific research and industrial procedures because of their ability to degrade polymers such as cellulose (20), polygalacturonate (16), galactomannans of legume seeds (11) or starch (14) and sugars such as maltose, cellobiose, lactose or raffinose (6). Some of their major industrial applications include production of high fructose corn syrups, baking, brewing, beverage clarification (12), production of low-lactose milk (13), reducing the content of raffinose in beat sugar molasses (19) and oligosaccharides of raffinose family in soybean milk (10). Industrial application of these enzymes, however, would only be feasible if they were available in large quantities at a competitive price. These glycosidases are also capable of hydrolyzing appropriate nonreducing terminal monosaccharide residues of carbohydrate units in glycoproteins and glycolipids (5) and may have an extensive application in the study of complex macromolecules (3). Studies on glycosidases are also of interest in medicine. It has been shown that in-vivo lactase replacement therapy improves lactose absorbtion and reduces gas production in the colon. In order to prevent the symptoms of lactose

intolerance in persons deficient in the endogenous enzyme, lactase has been added to milk and milk products, or taken by mouth before a meal containing dairy products. α-Galactosidase has also been added to legumes and other vegetables to reduce flatulance (15) caused by microbial fermentation of olygosaccharides such as raffinose and stachyose in the large intestine due to the lack of α-galactosidase in the human intestinal tract (10).

Glycosidases are produced by a number of filamentous fungi, such as *Aspergillus oryzae* (4,17), *Aspergillus niger* (5,23), *Aspergillus flavus* (1), *Aspergillus nidulans* (22), *Aspergillus ficuum* (28), *Aspergillus ornatus* (27), *Aspergillus fumigatus* (25), *Penicillium funiculosum* (20), *Penicillium brevicompactum* (16), *Penicillium notatum* (7). Altough glycosidases from some members of *Aspergillus* species have been intensively studied, enzymes produced by *Aspergillus flavipes* have received comperatively little attention.

The aim of the present study was to identify cultural conditions for production of extracellular α- and β-glucosidases, α- and β-galactosidases and α-amylase by *Aspergillus flavipes*.

Materials and Methods

Chemicals

Melibiose (Fluka 63630), cellobiose (Fluka 22150), raffinose (Fluka 83400), maltose (Merck 5911) and starch (Merck 1252) were used as carbon source. For enzyme assays p-nitrophenyl-α-D-galactopyranoside (Fluka 73653), p-nitrophenyl-β-D-galactopyranoside (Fluka 73670), p-nitrophenyl-α-D-glucopyranoside (Fluka 73673), p-nitrophenyl-β-D-glucopyranoside (Fluka 73674) were used as substrate. p-Nitrophenol (Fluka 73560) was used as standard for enzyme activity tests. All other chemicals were of analytical grade.

Microorganism and Culture Conditions

The microorganism used in this study was a strain of *Aspergillus flavipes* ACTT 11013 provided by Department of Microbiology, Faculty of Medicine, Istanbul University, Turkey. The fungal strain was maintained on Sabouraud dextrose agar plates, incubated at 22°C for 7 days, and then stored at 4°C. Spor suspansion was prepared by adding sterile distilled water to the culture plates and scraping the surface gently with a sterilized wire loop. The suspension was diluted in water to give a final inoculum of 5×10^4 spores/ml.

In order to determine the optimum conditions for mycelial growth and enzyme production, the effect of some parameters such as incubation time, initial pH of the medium, and different carbon sources on the enzyme activity were investigated.

Effect of initial pH: The initial pH of culture medium was adjusted to different values using 1N hydrochloric acid or 1N sodium hydroxyde solution. Sampling was carried out every 24 h by aseptically withdrawing 1 ml of culture medium.

Effect of carbon sources: Carbon sources were added to the culture medium separately, and their effects on the production of glycosidases were investigated.

Production of enzymes at different induction times: 1 ml of the culture medium was pipetted out and the enzyme activity at standard assay conditions was examined at 24 h intervals during growth of the microorganism.

Composition of media and cultivation of microorganism: This experiment was done with the medium of various concentration of peptone, KH_2PO_4, $(NH_4)_2HPO_4$, $MgSO_4 \times 7H_2O$ and KCl. α-Glucosidase and β-glucosidase production were induced by adding 10 g of maltose and 4 g of cellobiose, respectively to the media consistent of (per liter) 5 g of peptone, 8 g of KH_2PO_4, 2 g of $(NH_4)_2HPO_4$, 0.5 g of $MgSO_4 \times 7H_2O$. The medium used for the α-galactosidase production contained (per liter): 15 g of peptone, 15 g of KH_2PO_4, 5 g of $MgSO_4 \times 7H_2O$, 5 g of KCl; supplemented with 2 g of melibiose as a carbon source. α-Amylase was produced using a medium with the following composition (per liter): 5 g peptone, 7 g KH_2PO_4, 0.5 g $MgSO_4 \times 7H_2O$, 3 g $(NH_4)_2HPO_4$, and 10 g starch. After a volume of 100 ml of each medium was poured in 250 ml Erlenmeyer flasks, they were sterilized by autoclaving at 120°C for 30 minutes. The media were cooled to room temperature and inoculated with 1 ml of the spore suspansion, then incubated at 30°C .

Enzyme Assays

α-Galactosidase, β-galactosidase, α-glucosidase and β-glucosidase activities were assayed by measuring the amount of p-nitrofenol

released from p-nitrophenyl-α-D-galacto-pyranoside, p-nitrophenyl-β-D-galactopyranoside, p-nitrophenyl-α-D-glucopyranoside, p-nitropı-henyl-β-D-glucopyranoside, respectively. 100 µl of enzyme solution was incubated with 100 µl of 20 mM p-nitrophenyl D-glycopyranoside and 100 µl of Mc Ilvaine buffer (pH 4.8) at 37°C for 30 minutes. The reaction was stopped by adding 3 ml of 0.2 M Na_2CO_3 solution. The extinction of the solution was measured at 420 nm by using a Shimadzu UV-1208 spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme which liberated 1 µmol of p-nitrophenol per minute under assay conditions described.

α-Amylase activity was determined by using the method of Somogyi (18) and Nelson (24). A 200µl of enzyme sample was added to 200 µl of substrate solution which was prepared freshly by diluting 1% starch solution to 1:1 proportion with phosphate buffer (pH 5.9) and the mixture was incubated at 37 °C for 10 min. The enzymic reaction was stopped by adding 1 ml of Somogyi reagent. After an incubation at 100°C in a boiling-water bath for 20 min., the assay tubes were allowed to cool and 1 ml of Nelson reagent was added, followed by 10 ml of water. After 10 min the stabilized blue coloration was read spectrophotometrically at 520 nm against suitably prepared blancs. The enzyme activity unit was calculated as the number of µmol of 1,4-glycoside linkage hydrolysed per minute in each ml of the reaction medium.

Results and Discussion

In this study we aimed to find an active and more readily available source for the production of some glycosidases. Several α- and β-galactosidases, α-amylases, α- and β-glucosidases of mold origin have been intensively investigated, but most of the studies described in literature deal with the isolation and characterization of intracellular enzymes. Extracellular enzyme secretion is important in industrial applications. The increased number of recovery steps for intracellular enzymes including cell disruption, removal of other cellular debris, and a greater need for extended purification of the material can decrease the yield of a desired protein (21). Extracellular glycosidase production and purification has been reported for *Aspergillus niger* (2,23,26), *Aspergillus oryzae* (4,10), *Aspergillus tamarii* (9), *Penicillium brevicompactum* (16), *Penicillium notatum* (7). From this study, it was concluded that the α- and β-glucosidases, α-galactosidase and α-amylase from *Aspergillus flavipes* are inducible and that is an economical advantage for practical applications. Supernatant from culture of *Aspergillus flavipes* grown on lactose or polygalacturonate had not any detectable extracellular β-galactosidase activity. The failure to induce extracellular β-galactosidase production during growth on lactose has also been demonstrated for strains of *Aspergillus oryzae* and *Scropulaiopsis* sp. (16), *Penicillium brevicompactum* and *Aureobasidium pullulans* (17).

In the present study, optimum conditions for *Aspergillus flavipes* glycosidases were investigated. In order to determine the suitable method of cultivation, a preliminary experiment was carried out to examine the effect of the initial pH of the medium, the incubation time necessary for maximal enzymic activity and the composition of medium.

Figure 1 shows the time course analysis of culture of *Aspergillus flavipes* grown on various carbon substrates. The highest yield of enzyme was obtained after 6 days for α-amylase, 13 days for α- galactosidase, 12 days for β-glucosidase and 11 days for α-glucosidase

A medium containing salts, peptone and yeast extract was found to be the best for each enzyme production. α- and β-Glucosidases, α-galactosidase and α-amylase production was induced by various sources of carbon and comparative experiments were conducted using either melibiose or raffinose, lactose or

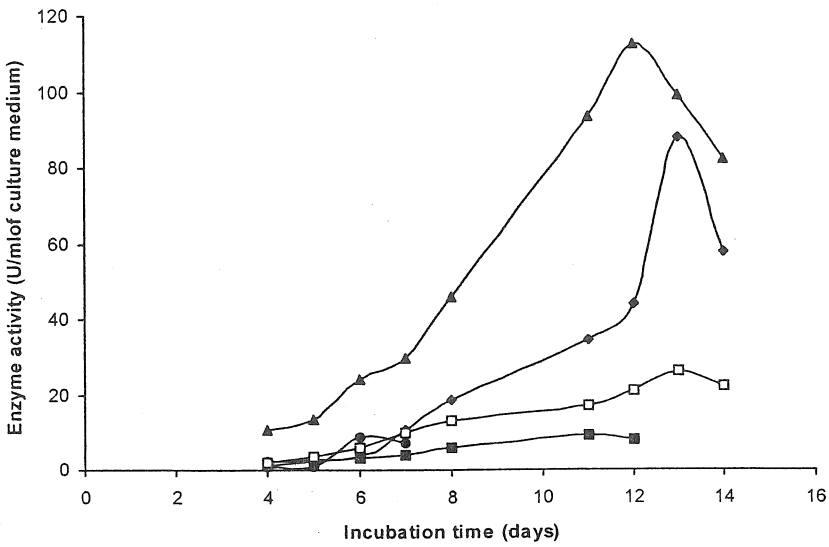

Fig.1. Time course of culture of *Aspergillus flavipes* grown on cellobiose —□—; melibiose - —◆—; raffinose - —*—; maltose - —■— ; starch - —●—

polygalacturonate, cellobiose or carboxymetylcellulose, respectively. Maximum yield of β-glucosidase (112.5 U/ml), α-glucosidase (9.1 U/ml) and α-amylase (3.5 U/ml) was obtained with cellobiose, maltose and starch, respectively. High α-galactosidase activity (88 U/ml) was detected in the culture supernatant when the mold was grown in a medium containing melibiose as a sole carbon source. Raffinose was found to be a weak inducer of α-galactosidase. A value of 26 U/ml of α-galactosidase activity was found using raffinose containing media. The enzyme productivity is higher than the productivity of *Aspergillus niger* (25) when this culture was grown on wheat bran or rice bran (0.125 U/ml or 0.08 U/ml).When the strains were compared according to their α-galactosidase production, *Aspergillus flavipes* showed better activity then the others.

An examination of the initial pH of the culture medium between 2 and 8 indicated that these hydrolytic enzymes had an optimal pH of 6 for α- and β- glucosidases activities, pH 6.5 for α-amylase activity and pH 5 for α - galactosidase activity.

Consenquently, the finding of our study may play a useful role in setting up the optimal conditions for a large-scale production of glycosidases. Extracellular α-amylase and α-glucosidase reported in this study showed very low activity, it is clear that further studies are necessary to optimize the conditions for cultivation of *Aspergillus flavipes* to obtain more yield of these enzymes. α-Galactosidase and β-glucosidase obtained from *Aspergillus flavipes* seems to have an excellent activity and could be used in the hydrolysis of raffinose and cellobiose, respectively.

References

1. Abou-Zeid, Alaa M.: Microbios 89 (358), 55 (1997)
2. Agnantiari, G., Christakopoulos, P., Kekos, D., Macris, B.J.: Acta Biotechnol. 11 (5), 479 (1991)

3. Agrawal, K.M.L., Bahl, O.P.: J. Biol. Chem. 243, 103 (1968)
4. Annunziato, M.E., Mahoney, R.R., Mudgett, R.E.: J.Food Sci. 51 (5), 1370 (1986)
5. Bahl, O.P., Agrawal, K.M.L.: J.Biol.Chem. 244, 2970 (1969)
6. Barnett, J.A.: Adv. Carbochydr. Chem. Biochem. 39, 347 (1981)
7. Berkkan, H.: Acta Pharmaceutica Turcica 29, 17 (1987)
8. Brown, J.A., Collin, S.A., Wood, T.M.: Enzyme Microb. Technol. 9, 176 (1987)
9. Civas, A., Eberhard, R., Le Dizet, P., Petek, F.: Biochem. J. 219, 857 (1984)
10. Cruz, R., Batistela, J.C., Wosiacki, G.: J. Food Sci. 46, 1196 (1981)
11. Dea, I.C.M., Morrison, A.: Adv. Carbohydr. Chem. Biochem. 31, 241 (1976)
12. Dziezak, J.D. : Food Technology 45, 78 (1991)
13. Hosinger, V.H., Kligerman, A.E.: Food Technology 45, 92 (1991)
14. Guilbot, A., Mercier, C.: Starch. In: Aspinall, G.O. (Ed.), The polysaccharides, pp 209-82, Academic press, New York, 1985
15. Martindale, W. (Ed.). The Extra Pharmacopoeia, 30th Ed., pp 1421, 1474, The Pharmaceutical Press, London, 1993
16. Mc Kay, A.M.: Letters in Applied Microbiology. 13, 71 (1991)
17. Mc Kay, A.M.: J. Food Sci. 56 (6), 1749 (1991)
18. Nelson, N.: J. Biol. Chem. 153, 375 (1944)
19. Obara, J., Hashimoto, S.: Sugar Technol. Rev. 4, 209 (1977)
20. Parr, S.R.: Enzyme Microb. Technol. 5, 457 (1983)
21. Penet, C.S.: Food Technology 45, 98 (1991)
22. Rios, S., Pedregosa, A.M., Monistrol, I., F., Laborda, F.: FEMS Microbiol. Lett. 112 (1), 35 (1993)
23. Somiari, R.I., Balogh, E.: Enzyme Microb. Technol. 17, 311 (1995)
24. Somogyi, M.: J. Biol. Chem. 195, 19 (1952)
25. Wase, D.A.J., Raymahasay, S., Wang, C.W.: Enzyme Microb. Technol. 7, 225 (1985)
26. Watanabe, T., Sato, T., Yoshioka, S., Koshijima, T., Kuwahara, M. Patol.: Biochem. 209, 651 (1992)
27. Yeoh, H.H.,Tan, T.K, Koh, S.K.:Appl. Microbiol. Biotechnol. 25 (1), 25 (1986)
28. Zapater, I.G., Ulah, A.H.J., Wodzinski, V.M.: Prep. Biochem. 20, 263 (1990)

Accepted: 23.12.1999