

Polygalacturonases Produced Under Solid State and Submerged Fermentation Conditions by Two Strains of *Aspergillus foetidus*

[*Aspergillus foetidus* 'a Ait İki Suş Tarafından Katı Kültür ve Batık Kültür Fermentasyon Koşullarında Üretilen Poligalakturonazlar]

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ABSTRACT

Objectives: Polygalacturonases produced by two strains of *Aspergillus foetidus* (EGEK145, EGEK635) under solid state and submerged conditions were investigated for some of their biochemical characteristics.

Materials and Methods: Solid state fermentation showed 341 and 297 times higher production of polygalacturonases of EGEK145 and EGEK635 respectively as compared to submerged fermentation. As was shown by isoelectric focusing, only some acidic forms of polygalacturonases were produced during submerged fermentation instead of the broad spectrum of enzyme forms obtained during solid state fermentation. Extraction of proteins from solid state conditions was followed by desalting on Sephadex G-25 column, CM-Sephadex C-50 and Concanavalin A - Sepharose purification steps and determination of molecular mass of polygalacturonases and exopolygalacturonases on Superose 12 column.

Results and Conclusions: Some determined biochemical features of polygalacturonases mixture produced by both strains under solid state conditions are: pH optima: 4.8 for the major form and 4.4 for the minor form; temperature optima as 30° C and 35° C; V_{max} : $22.62 \pm 0.96 \mu\text{mol}/\text{min}$ and $153.84 \pm 0.77 \mu\text{mol}/\text{min}$, K_m : $4.52 \times 10^{-5} \pm 0.24 \times 10^{-5} \text{ mol}/\text{L}$ and $4.62 \times 10^{-5} \pm 0.03 \times 10^{-5} \text{ mol}/\text{L}$ for EGEK145 and K635, respectively. Molecular masses of exopolygalacturonase and polygalacturonase were 54 and 31 kDa, respectively for EGEK145. In addition, thermal stabilities, action pattern on pectate, their glycoprotein character were determined and compared.

Key Words: *Aspergillus foetidus*, polygalacturonases, solid state fermentation, biochemical characterisation

ÖZET

Amaç: *Aspergillus foetidus*'un iki suşu (EGEK145, EGEK635) tarafından katı kültür ve batık kültür fermentasyon koşullarında üretilen poligalakturonazların bazı biyokimyasal özellikleri incelenmiştir.

Yöntemler: Poligalakturonazların üretiminde katı kültür fermentasyonu batık kültür fermentasyonuna göre, sırasıyla EGEK145 için 341 kat, EGEK635 için 297 kat fazla üretim sağlamıştır. İzoelektrik odaklama ile gösterildiği üzere, katı kültür fermentasyonu sırasında üretilen geniş spektrumlu enzim formlarının aksine batık kültür fermentasyonu ile sadece bazı asidik formlar üretilmiştir. Katı kültür koşullarında üretilen proteinlerin ekstraksiyonunu, Sephadex G-25 kolonunda tuzsuzlaştırma, CM-Sephadex C-50 ve Concanavalin A- Sefaroz saflaştırma adımları ve Superose 12' de poligalakturonaz ve ekzopoligalakturonazların moleküler kütlelerinin belirlenmesi izlemiştir.

Bulgular ve Sonuç: Her iki strain tarafından katı kültür fermentasyon koşullarında üretilen poligalakturonaz karışımının bazı belirlenen özellikleri sırasıyla EGEK145 ve EGEK635 strainleri için, majör optimum pH 4.8 ve minor optimum pH 4.4 olarak, optimum sıcaklık 30 °C ve 35 °C, V_{max} değerleri $22.62 \pm 0.96 \mu\text{mol}/\text{dak}$ ve $153.84 \pm 0.77 \mu\text{mol}/\text{dak}$, K_m değerleri $4.52 \times 10^{-5} \pm 0.24 \times 10^{-5} \text{ mol}/\text{L}$ ve $4.62 \times 10^{-5} \pm 0.03 \times 10^{-5} \text{ mol}/\text{L}$ 'dir. EGEK145 için ekzopoligalakturonaz ve poligalakturonaz moleküler ağırlıkları 54 ve 31 kDa'dır. Buna ilaveten termal kararlılıkları, pektat üzerinde etki tarzı, glikoprotein karakteri belirlenmiş ve karşılaştırılmıştır.

Anahtar Kelimeler: *Aspergillus foetidus*, poligalakturonazlar, katı kültür fermentasyonu, biyokimyasal karakterizasyon

Introduction

Pectin is a complex heteropolysaccharide found in middle lamella of higher plants. It accounts 0.5 - 4 % of weight of fresh material. When the tissue is ground, pectin is found in the liquid phase (soluble pectin) causing an increase in viscosity and in the pulp particles, whereas other pectin molecules remain bound to cellulose fibrils by means of side chains of hemicellulose and thus facilitate water retention (1). Due to its complex structure a complex enzyme system comprising numerous enzymes are involved in its degradation. (i) depolymerases that remove the acetyl and methyl groups from pectin, (ii) depolymerases that cleave the backbone of pectin. The main classes of depolymerizing enzymes, collectively called pectinases are polygalacturonases, exopolygalacturonases, rhamnogalacturonases, pectin lyases, pectat lyases and rhamnogalacturonan lyases (2). Pectolytic enzymes are produced mainly by fungi and bacteria. They have widespread applications in food industry, wastewater treatment, textile industry, fruit softening and plant infection processes (3, 4). Since they hold GRAS (generally regarded as safe) status for food industry, commercial production of polygalacturonases are mainly by submerged fermentation (SmF) by using *Aspergillus aculeatus* and *A. niger* which belong to taxonomic section "*Aspergillus Section Nigri*", (5).

Production of microbial metabolites by solid state fermentation (SSF) using agrowastes is a growing area of biotechnology (6). This system can be explained as the cultivation of microorganisms on solid materials without the presence of free liquid in contrary to submerged fermentation conditions. It has been proved that SSF offers serious advantages over SmF such as higher productivity and higher enzyme titers due to absence or resistance to catabolic repression, higher oxygen diffusion and oxygen levels at solid-air interface, more suitable growth conditions for fungi than bacteria and yeasts and lower bacterial contamination risk by the lower moisture level (7). In a previous study, it was shown that *A. foetidus* EGEK635 and EGEK145 strains (belong to same section as stated before) were able to grow and produce pectolytic enzymes on agrowastes under SSF conditions (8). Twenty times higher enzyme activities were obtained by utilizing SSF in comparison to SmF after improvement of fermentation conditions on the same production medium.

Production of enzymes from agrowastes by SSF such as lemon and apple peel, sugar cane bagasse, sugar beet pulp or coffee pulp can be effective because they contain large amounts of cellulose, hemicellulose and pectin which could serve as inducers for cellulases, xylanases and pectinases, respectively (9).

In spite of numerous studies have been carried on mold originated PGases, numerous articles and patents have recently reported the new pectolytic enzymes with putative applications, notably on fruit juice application.

It is obvious that PGases differ in molecular sizes, pH optima, and other characteristics independent of their source. This difference is also related to fermentation type (10). Since applications of pectolytic enzymes in various fields are widespread, it is important to understand nature and properties of these enzymes for efficient and effective usage. In this study, PGases from strains of *Aspergillus foetidus* produced by SSF on regional agrowastes were isolated and some of their biochemical characteristics were determined. Fermentation medium was a mixture in a best suited ratio of sugar beet pulp and wheat bran which was determined as suitable in our previous study (8).

Material And Methods

Strains and fermentation conditions

The strains were formerly isolated from vineyards and deposited at microfungi culture collection at Ege University, Bioengineering Department, Izmir, Turkey.

Strains were precultivated at Potato dextrose agar (PDA) for five days for spore production and they were harvested by scraping into sterile 0.01 % Tween 80 solution. For enzyme production by fermentation, 2 ml of spore suspensions (10^7 spores/ml) were inoculated into both SSF and SmF conditions. Media were incubated at 28°C in dark.

SSF medium was composed of sugar beet pulp and wheat bran in 2:1 ratio and moistened with 75% moisture level with a mineral solution containing (w/v): 1% $(\text{NH}_4)_2\text{SO}_4$; 0.1% K_2HPO_4 ; 0.1% $\text{Mg}(\text{SO}_4) \cdot 7 \text{H}_2\text{O}$ and 0.001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. All media were incubated for three days, statically.

SmF medium was also contained same mineral solution as mentioned above for SSF. The concentration of pectin used in cultivation medium was 1.3 % (w/v). For each strain, 4 x 1000 ml media were incubated for six days, statically.

Commercial citrus pectin (GENU Pectin, Denmark) was purified as previously described (11). Thus degree of esterification of purified pectin was 45% and contained 87% uronic acid in dry matter.

Enzyme assay

The total PGase activity was assayed at 45 °C (temperature optima was measured at the range of 20-70 °C) in time intervals by measuring the increase of reducing groups (12). In the reaction mixture containing five aliquots of sodium pectate (0.2 g/dL in 0.1 M acetate buffer, pH 5.2) as a substrate (except the determination of pH optima where the substrates with pH values in the range of 3.6 - 5.6 were used) and one aliquot of protein solution with proper dilution. The total activity of the polygalacturonase/exopolygalacturonase system was expressed in μmol of reducing groups liberated within one minute per mg of extracellular protein precipitate and determined

by means of standard graph for D-galacturonic acid. After partial characterization of enzymes, 0.5 % (g/ml) pectate solution, pH 4.8 in 1:1 ratio with enzyme solution at 30 °C was used.

Protein determination

Protein concentrations were determined by Bradford method using BioRad reagent and bovine serum albumin (BSA) as standard (13).

Isolation of PGases

Preparation of crude protein extracts from fermentation medium

After incubation period, fermented medium under SSF conditions was extracted with 0.1 M Na-acetate buffer (pH 4.4) in 1:10 (w/v) ratio. Then extract was filtered by Whatman No:4 and centrifuged at 3622 xg for 10 minutes. Supernatant was used for further steps. From SmF conditions the cells were removed by filtration and used for enzyme assay and characterization.

Crude extracellular protein mixtures for the extracts were obtained by the precipitation of supernatant (from SSF) or filtrated pectin medium (from SmF) with ammonium sulfate (90% of saturation, 24h, 4 °C), filtration, dissolution of precipitate in small amount of water followed by precipitation with ethanol (1:4, 24h, 4°C). After partial desalting by dialysis against water, desalting of proteins was completed on Sephadex G-25 column. Thus obtained freeze-dried crude proteins were used for PGase activity assay as well as for the determination of multiple forms of this enzyme by isoelectric focusing (IEF).

Partial purification of PGases

Partial purification of PGases obtained from SSF conditions was provided on CM-Sephadex C-50 column (length 250 mm, diameter 25 mm) equilibrated with acetate buffer (0.05 M, pH 3.6). Proteins were eluted stepwise with 0.05 M acetate buffer, pH 3.8, 0.10 M acetate buffer, pH 4.4, 0.15 M acetate buffer, pH 4.8, 0.20 M acetate buffer, pH 5.2, and, finally, 0.20 M acetate buffer, pH 5.2 containing 1.0 M NaCl at a flow rate of 12 ml/h. Fractions were collected for every 30 min. The eluted

fractions were monitored for protein contents (at 280 nm) and enzyme activities.

The next step was provided on a Concanavalin A-Sepharose column (Pharmacia, Sweden) (length 25 mm, diameter 15 mm) in 0.1 M acetate buffer, pH 4.7 with addition of 0.1 M NaCl, 0.001 M MnCl₂ and 0.001 M CaCl₂ (fractions of 1 ml). 0.1 M Methyl- α -D-mannopyranoside (Sigma, Germany) in 0.1 M acetate buffer, pH 4.7, with 0.1 M NaCl was used as an eluting agent.

No further separation steps were performed. Some characteristics of purification were summarized on Table 1.

Characterization of PGases

pH and temperature optima were estimated by performing standard enzyme assay utilizing conditions within the range of 3.6-5.6 for pH and 24.5-70 °C for temperature.

The kinetic parameters (K_m and V_{max}) of PGases toward pectate (molecular weight 27 000) at pH 4.8 and 30 °C were obtained by double reciprocal Lineweaver-Burk plots utilizing substrate in the range of final concentrations of 3.7037×10^{-5} M, 2.9629×10^{-5} M and 2.2222×10^{-5} M.

Thermal stabilities of PGases were evaluated after 2 hours incubation of enzyme solutions at 30–60 °C followed with enzyme activity assay at 30 °C.

Storage stabilities at 4 °C of freeze-dried PGases as well as of PGases in 0.1 M acetate buffer, pH 4.8 were evaluated by activity assay provided in 7-days time intervals.

Viscosity measurements were provided during degradation of 0.5 % (g/ml) pectate solution in 0.1 M acetate buffer, pH 4.8 by extracellular PGases in an Ubbelohde viscosimeter. The action pattern of produced enzymes was determined by the correlation of viscosity decrease of polymeric substrate with the degree of its degradation (14).

Molecular mass evaluation was performed by gel permeation chromatography on a Superose 12TM, HR 10/30, column (FPLC device, Pharmacia, Sweden); fraction size: 0.5 ml/min; mobile phase: 0.05 M phosphate buffer, pH 7.0, containing 0.15 M NaCl. Molecular masses were approximately evaluated by a calibration curve obtained with a Molecular weight marker kit (Sigma) with the mo-

Table 1. Partial purification scheme for PGases produced under solid state conditions by *A. foetidus* EGK145 & EGK635

Steps	Total unite (U)	Total protein (mg)	Specific activity (U/mg)	Fold purification	Recovery (%)
Freeze-dried crude extract (198 mg) & (88 mg)	74.98 & 71.23	0.97 & 0.72	77.29 & 98.93	1 & 1	100 & 100
CM-Sephadex C-50	3.22 & 2.62	0.52 & 0.44	6.19 & 5.95	0.08 & 0.06	53 & 61
Concanavalin A-Sepharose column	2.24 & 1.83	0.10 & 0.10	22.4 & 18.3	0.28 & 0.18	10 & 14

lecular weight range of 12.5 – 700 kDa (14). These markers and their Mr values (in parenthesis) were as follows: Blue Dextran (1995262), Thyroglobulin (676083), Apoferritin (446683.6), β -amylase (199526.1), Alcohol dehydrogenase (151356.1), Bovine Albumin (66069.34), Carbonic anhydrase (28840.32), Cytochrome C (12302.69).

Exopolygalacturonase activity was distinguished by using 1 mM solution of digalacturonic acid in 0.1 M acetate buffer, pH 4.8.

SDS-PAGE for molecular mass analysis of proteins obtained after CM-Sephadex and ConA – Sepharose steps was performed in a Mini-Protean 3 Electrophoresis System (Bio-Rad Laboratories, California) under reducing conditions (with β -mercaptoethanol). The silver-staining method was used for band visualization as shown in Figure 8 (15). As the molecular size markers proteins in the range of 17 – 95 kDa (Serva) were utilized.

Ultrathin-layer isoelectric focusing (IEF) in polyacrylamide gels on polyester films was performed as described (16). Polygalacturonase activity was detected by the print technique with D-galacturonan (degree of polymerisation 10) dyed additionally with ruthenium red (Sigma, Germany) (17). As a standard, Test Mix 9 (Sigma) was used.

Results And Discussion

Generally, PGases characterized previously are mainly obtained by submerged cultivation of the strains or from commercial preparations. In this study PGases produced under SSF and SmF conditions by the two strains of *A. foetidus* have been compared and the main characteristics of those produced under SSF were investigated.

Crude protein precipitates obtained after cultivation under SSF showed 341 and 297 times higher total activity of PGases for strains EGEK145 and EGEK635 respectively than the activities of precipitates obtained after cultivation by SmF. The specific activities were higher for both strains (about five times). The reason was clarified by utilizing IEF with detection on PGase activity (Figure 1). As can be seen only strongly acidic forms of enzyme were produced on pectin medium during 6 days cultivation while the whole spectrum of PGases was produced when wheat bran and sugar beet pulp was used (ratio 1:2) for three days under SSF. This phenomenon can be explained due to the various pH conditions of cultivations because of known direct induction of individual PGase forms with pH (18).

The enzymes produced under SSF conditions were further characterized and these characteristics are summarized in Table 2. The major pH optimum of PGase mixture produced by both strains was 4.8 and the minor one corresponded to pH 4.4 (Figure 2). Temperature optima were 30°C and 35°C for EGEK145 and EGEK635, respectively (Figure 3). At 40 °C, both enzymes kept 94 % of their activities, but the thermal stabilities of both were comparably low. By two hours incubation at this

temperature they kept only 10.7 (EGEK145) and 33.3 % (EGEK635) of initial activity (Figure 4). Therefore 30 °C can be recommended as the most suitable working temperature for these enzyme preparations. PGases were very unstable when they were stored in 0.1 M acetate buffer, pH 4.8, at 4°C. Activities of the enzymes were reduced to 50 % of initial activity after 9 days (for EGEK145) or 11 days (for EGEK635) upon storage. Freeze-dried PGases are 100 % stable at this temperature.

The action pattern of PGase mixture produced by both strains was determined by correlating the viscosity decrease with the degree of degradation of pectate as a substrate (Figure 5). The main polygalacturonase pattern was determined to be random.

Molecular masses of polygalacturonase and exopolygalacturonase in protein precipitate produced by EGEK145 strain were evaluated by gel permeation chromatography utilizing Superose 12 column and calibration proteins (Figure 6A). Digalacturonic acid was used for specific exopolygalacturonase determination (not cleaved by endo-acting enzyme). Molecular masses of exopolygalacturonase and polygalacturonase were 54 and 31 kDa, respectively. Fraction numbers and log Mr values of calibration proteins were also given in Figure 6B. PGase preparations with both endo- and exo- activity are particularly important for efficient hydrolyzation of natural pectin sources since they act as a substrate for both types of enzymes.

K_m values determined on pectate as a substrate were $4.52 \pm 0.24 \times 10^{-5} \times 10^{-5}$ mol/L and $4.62 \pm 0.03 \times 10^{-5}$ mol/L and V_{max} values were 22.62 (+/- 0.96) μ mol/min and 153.84 (+/- 0.77) μ mol/min for EGEK145 and K635, respectively (Figure 7A-B). These values showed typical high affinity of PGases towards pectate.

Previous studies have shown multiple forms of PGases produced by different strains of *Aspergillus*. Multiple forms of polygalacturonases (PGI, PGII and PGIII) from *A. carbonarius* (commercial preparation on wheat bran by SSF) with molecular weights of 61, 42 and 47 kDa have been reported (19). Their optimum for pH were 4.0, 4.1 and 4.3, and for temperature 55°C, 50 °C and 55 °C, respectively. Multiple forms of PGases we observed in this study, too, especially in the case of SSF cultivation. Unlike variability in isoelectric points only one molecular mass of exopolygalacturonase and one for polygalacturonase was found. For this reason the partial purification of enzymes was performed by ion-exchange chromatography on CM-Sephadex C-50 column. PGases were released only at pHs 4.4 (about 30 % of total activity) and 4.8 (about 70 %) without separation of individual enzyme forms. About ten times higher specific activities were obtained after this step. Other possibility to purify these enzymes is based on the interaction of glycoproteins with Concanavalin A (ConA) bound on some suitable matrix. ConA – Sepharose was used. All

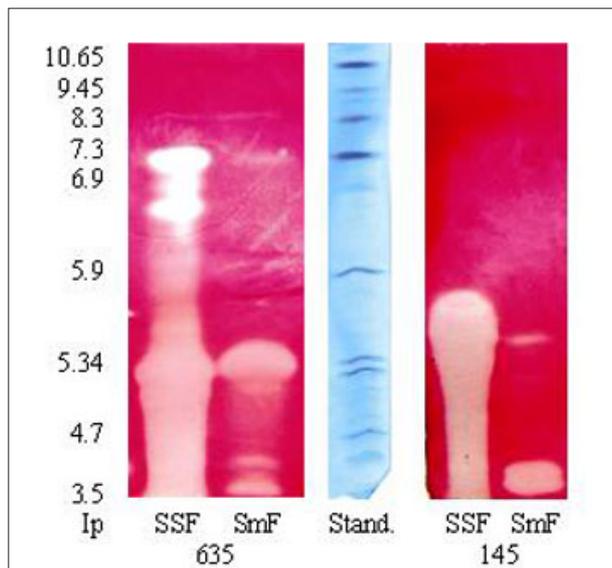


Figure 1. Isoelectric focusing in ultrathin polyacrylamide layers (pH gradient 3-10) of multiple forms of polygalacturonases produced by *Aspergillus foetidus* strains EGEK635 and EGEK145. SSF and SmF-conditions of cultivation. 1 ml of concentrated samples applied. Polygalacturonase activity detected by the print technique with a specific substrate (D-galacturonan with degree of polymerisation 10) dyed additionally with ruthenium red.

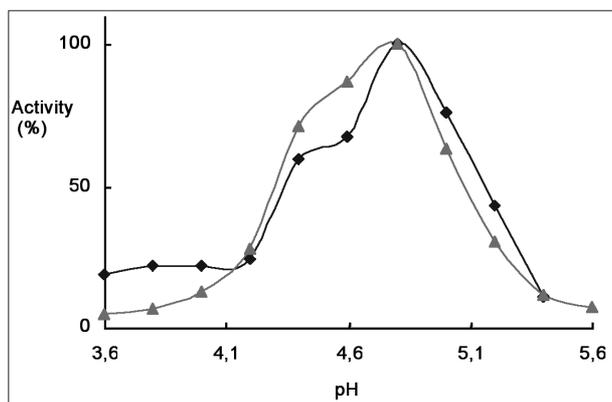


Figure 2. pH optima of PGases of *Aspergillus foetidus* strains EGEK145 (◆) and EGEK635 (▲) produced under solid state conditions

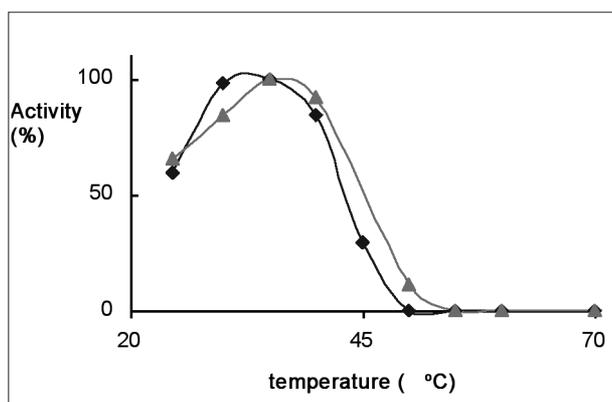


Figure 3. Temperature optima of PGases of *Aspergillus foetidus* strains EGEK145 (◆) and EGEK635 (▲), produced under solid state conditions

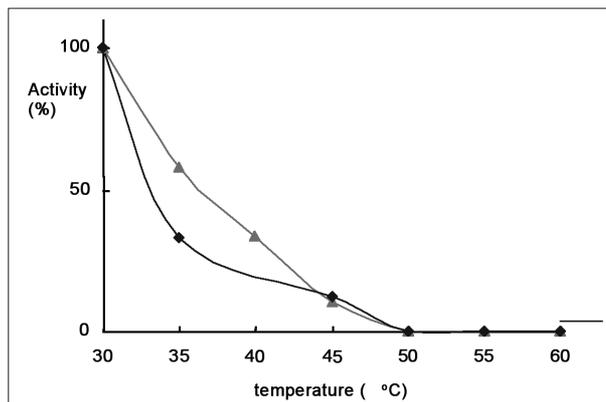


Figure 4. Thermal stabilities of PGases of *Aspergillus foetidus* strains EGEK145 (◆) and EGEK635 (▲) produced under solid state conditions

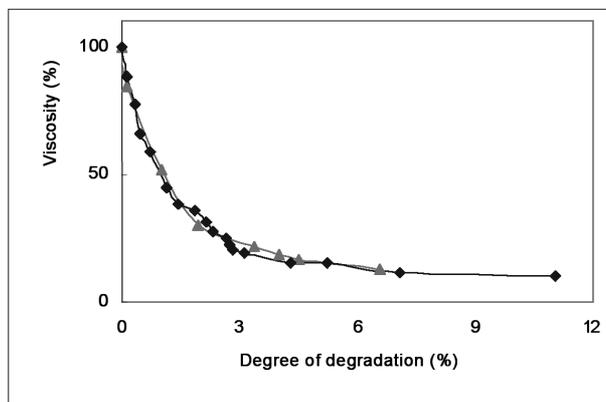


Figure 5. Action pattern of PGases with 0.5% pectate solution (w/v, in 0.1 M acetate buffer, pH 4.8) produced by *Aspergillus foetidus* strains EGEK145 (◆) and EGEK635 (▲) under solid state conditions.

forms of PGases interacted with ConA showing their glycoprotein character. About 60 % of contaminating proteins which do not interact with lectin was removed. SDS-PAGE with silver staining showed protein bands corresponding to molecular masses of 54 and 31 kDa but a considerable amount of protein contamination was still present (Figure 8).

Five types of pectolytic enzymes (one of which was a PGase) from *A. niger* MIUG16 produced by solid state fermentation have been purified and characterized (20). Some biochemical properties were determined as 4.6 for pH optimum, 40 °C for optimum temperature and rapid activity loss beyond pH 5.0. K_m and V_{max} values were 0.94 g/L and 3114.3 U/mg, respectively.

PGases from *A. awamori* IFO 4033 obtained by submerged cultivation were investigated biochemically. Optimal values for their activity were determined as 5.0 for pH, 40°C for temperature, thermal stability as around 30 °C. Molecular mass according to SDS-PAGE and gel filtration was 41 kDa (21).

New three exo-acting PGases named PGXA, PGXB, PGXC from *A. niger* CBS 513.88 have been identified by transcriptional profiling and their pH optima on pectate (0.25 %) were determined as 3.5-4.0, 4.0-4.5, 3.5-4.0, respectively (22).

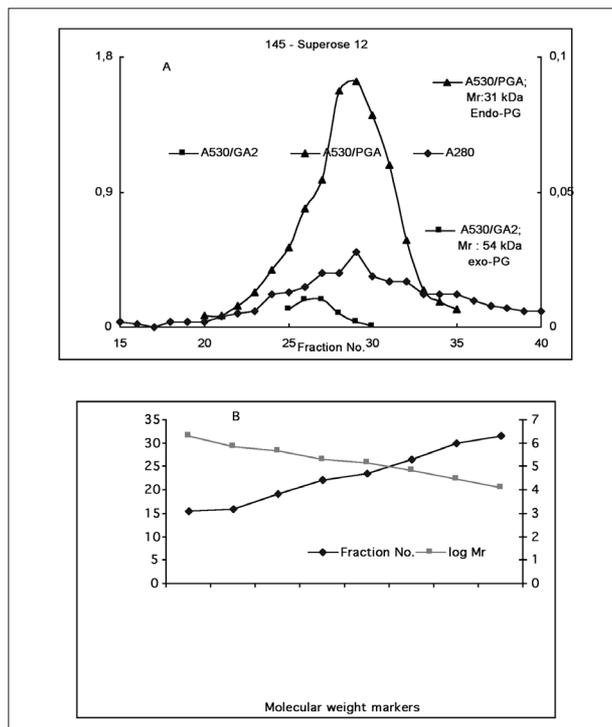


Figure 6. A- Molecular mass determination of PGases produced by EGEK145 utilizing gel-permeation chromatography on Superose 12TM (HR 10/30, column (FPLC device), mobil phase 0.05 M phosphate buffer, pH 7.0, containing 0.15 M NaCl at a flow rate 30 ml/h. Proteins expressed as A280 (◆), activity on 0.5% pectate(w/v), pH 4.8, and absorbances of PGases on polygalacturonic acid (▲, A530/GA), on 1 mM digalacturonic acid (■, A530/GA2) pH 4.8. Molecular masses were approximately evaluated from a calibration curve obtained with a molecular weight marker kit (Sigma) with the molecular weight range 12.5 – 700 kDa. B- Molecular weight markers with fraction numbers on Superose column and their log Mr values.

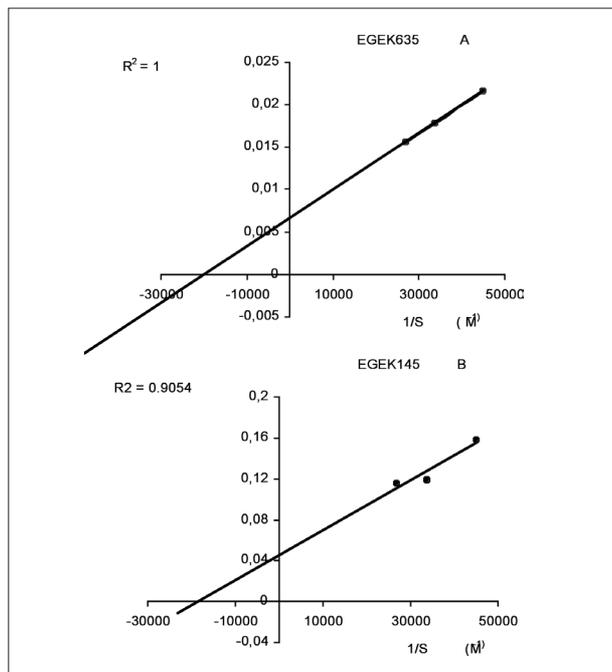


Figure 7. Plot of enzymatic reaction velocity ($1/v$) versus the reciprocal of the substrate concentration ($1/[S]$) for PGases of A- EGEK635 and B- EGEK145 with pectate as substrate at pH 4.8 and 30 °C utilizing substrate in the range of concentrations in 3.7037×10^{-5} , 2.9629×10^{-5} and 2.2222×10^{-5} mol/L.

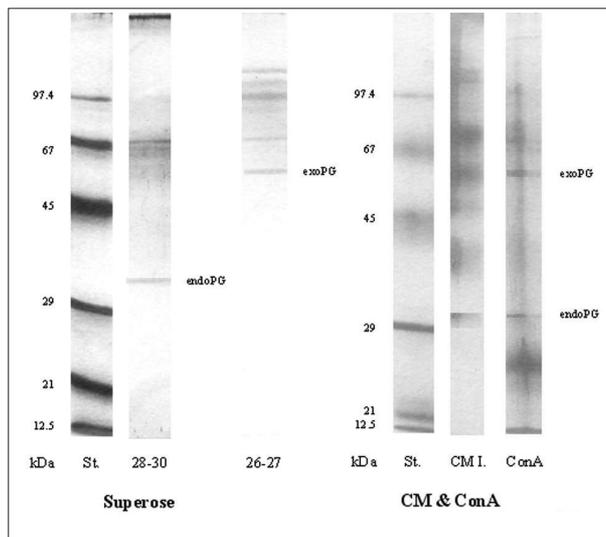


Figure 8. SDS-PAGE patterns of endo and exoPGases of *Aspergillus foetidus* EGEK145 after separation procedure steps on CM (CM-Sephadex C-50), ConA (Concanavalin A-Sepharose) and Superose columns. St: molecular weight markers.

As represented by the results of this study, the biochemical characterization of PGases described in this work showed many similarities to enzymes produced by other species of the genus *Aspergillus*. Biochemical characterization of an enzyme is a general requirement for their further successful utilization for industrial purposes.

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