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Full Length Research Paper

Physico-chemical characteristics of immobilized polygalacturonase from *Aspergillus niger* (SA6)

Mohammed L. Buga[1]*, Sani Ibrahim[2] and Andrew J. Nok[2]

[1]Raw Materials Research and Development Council (RMRDC), Plot 427, Aguiyi-Ironsi Street, Maitama District, P.M.B. 232, Garki, Abuja, Nigeria.
[2]Department of Biochemistry, Ahmadu Bello University, Samaru, Zaria, Nigeria.

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Polygalacturonase (PG) was isolated from *Aspergillus niger (A. niger)* (SA6), partially purified, characterized and immobilized by entrapment using calcium alginate. The polygalacturonase showed two bands on sodium dodecyl sulfate polyacryamide gel electrophoresis (SDS-PAGE) suggesting an "endo and exo" polygalacturonase with apparent molecular weights of 35 and 40 KDa, respectively. The enzyme was purified 9 fold with a yield of 0.18% and specific activity of 246 µmole/min/mg. The apparent K_M and V_{max} of the immobilized polygalacturonase were11.1 mg/ml and 1.65 µmole/min/mg, respectively. The optimum pH and optimum temperature of the immobilized polygalacturonase were 4.5 and 40°C, respectively. Immobilized polygalacturonase exhibited more stability to changes in pH than the temperature. The activity of the immobilized polygalacturonase reduced to 34.56 and 14.81% of the initial activity in the second and third catalytic cycles, respectively. The half life of the enzyme and the activity lost per minute on thermal storage were 10 min and 0.0213 µMole of D-galacturonic acid.

Key words: Polygalacturonase, *Aspergillus niger*, pectinases, enzymes.

INTRODUCTION

Pectinases (EC. 3.2.1.15 and EC. 3.2.1.67) endo and exopolygalacturonase, (EC. 4.2.2.10), polymethylgalacturonate lyase and (EC. 3.1.1.11), pectin esterase are a group of enzymes that can degrade pectin containing substrate or modify it during fruit ripening. Pectinases are produced by fungi, yeast, bacteria, protozoa, insects, nematodes and plants (Ahmed et al., 1997; Solis et al., 1997; Whitaker, 1991). Pectinases are used in the food industry for improvement of cloud stability in fruits and vegetable nectars, modification of pectins and processing of natural fibres in textile industry and haze removal from wines (Akin et al., 2001; Baker and Bruemmer, 1972; Baracat et al., 1991; Bauman, 1981; Csiszar et al., 2001; Gupta et al., 1993; Kilara, 1982; Rombouts and Pilnik, 1978; Sawada and Ueda, 2001).

Production of pectinases can be done by both solid state cultures and submerged fermentation techniques. However, production of pectinase by *Aspergillus* strains was observed to be higher in solid state fermentation than in sub-merged process (Acuna-Arguelles et al., 1995; Akinola and Olatunji, 2000; Maldonado and Strasser De Saad, 1998; Ronald et al., 1997; Sebastain et al., 1996; Solis-pereira et al., 1996; Tuttobello and Mill, 1961).

Aspergillus niger is the main micro organism used for the production of pectinases in the food industry and has been found to be safe owing to its wide use (http:// www.epa.gov/biotech.rule/pubs/fra/fra006.htm).

Pectin, the natural substrate of pectinase is synthesized in the golgi bodies of plants and forms a matrix in which the hemicellulose polysaccharides of the plant cell are embedded. It is broken down by pectinase to pectinic acid and finally pectic acid. During this chemical breakdown, the fruit gets softer (ripens) as the cell wall degenerates (McGraw, 1987).

Enzyme immobilization in the food processing industry

*Corresponding author. E-mail: mblawal@hotmail.com.

Abbreviations: DNS, Dinitrosalicylic acid; **FPLC,** fast protein liquid chromatography; **DEAE,** diethylaminoethyl; PG, polygalacturonase.

helps in the development of continuous process, economic organization of operations, automation and decrease of labor, greater control over reactions, high volumetric productivity and low residence time (Hartmier, 1988). Immobilized enzymes give products that are relatively pure, which is an important factor especially in food processing and pharmaceutical industries where contamination could be of serious toxicological, sensory or immunological consequences. Enzymes can be immo-bilized on a variety of natural and synthetic supports. The choice of support and/or technique depends on the nature of the enzyme, its substrate and its application. Attempts have been made to immobilize commercial pectinase preparations on various supports for fruit processing and endo-polygalacturonase (Deviet et al., 2004; Pier et al., 2004; Sohel et al., 2007). However, despite its potentials, immobilization of polygalacturonase (PG) isolated from a local strain of *Aspergillu*s (SA6) has not been attempted since commercial pectinases are often mixtures of cellulases, hemicellulases and pro-teases hence, they are poorly characterized. This would provide empirical data for future research. We report for the first time immobilization and characterization of polygalacturonase isolated from *A. niger* (SA6).

MATERIALS AND METHODS

All the reagents used in this study were of analytical grade purchased from Sigma Chemical Company representatives in Germany.

Microorganism

A. niger (SA6) strain was collected from the culture bank of Crop Protection Department of the Institute for Agricultural Research (IAR), Ahmadu Bello University, Zaria, Nigeria. The culture was maintained on potato dextrose agar and sub-cultured periodically throughout the duration of the research.

Preparation of media and cultivation of *A. niger (SA6)*

Exactly 200 g of potato was sliced, cut and boiled. It was filtered with muslin cloth and the volume made up to 1000 ml. Exactly 20 g each of dextrose and agar were added, boiled and autoclaved at 121°C for 15 min. After cooling, about 5 g of streptomycin powder was added to prevent bacterial contamination (http: en.wikibooks. org/Potato Dextrose Media Preparation).

Cultivation of the *Aspergillus* was carried out in pre-weighed 250-ml Erlenmeyer flasks covered with aluminum foil containing 5 g of wheat germ and 7.5 ml of 0.4 M HCl. The medium containing flasks were autoclaved at 121°C for 15 min. After cooling, each flask was weighed and water loss during autoclaving was calculated (Sebastain et al., 1996).

Inoculation of *A. niger (SA6)*

Ten milliliter (ml) of conidial suspension (approximately 10^7 spores/g of dry substrate), which was obtained from a 7-day old agar slant suspended in autoclaved Tween 80 solution was used to inoculate the substrate in a sterile chamber.

After inoculation, the contents were carefully mixed and the flasks placed in a humid cultivation room at 30°C under static condition for 3 days according to the method of Sebastian et al. (1996).

Preparation of crude polygalacturonase

After cultivation, cultures were suspended in 40 ml of distilled water stirred for 10 min and the pH measured. This was followed by the addition of 40 ml of 0.05 M acetate buffer pH 5.0 and the mixture left to stand for 10 min. The mixture was filtered through a muslin cloth and Whatman No. 1 filter paper and the solid material retained on the filter paper was extracted again with 20 ml of 0.05 M acetate buffer pH 5.0.

Both extracts were pooled, clarified by centrifugation at 5000 xg for 30 min and brought to a total volume of 100 ml with 0.05 M acetate buffer. The extract (crude enzyme) was kept at 4°C until used (Sebastain et al., 1996).

Ultrafiltration of crude polygalacturonase

The crude polygalacturonase was concentrated using Amicon equipment with cellulose acetate membrane in order to remove low molecular weight proteins of 10,000 KDa and below.

Ammonium sulphate precipitation

The crude polygalacturonase was precipitated with gentle stirring at 40 - 80% saturation of solid ammonium sulphate. It was centrifuged at 10,000 ×g for 15 min. The precipitate was re-dissolved in 0.05 M acetate buffer pH 5.0 and dialyzed overnight against the same buffer to remove low molecular weight substances and other ions that may interfere with the enzyme activity (Dixon and Webb, 1964).

Determination of protein content

Twenty micro litre (µl) of the enzyme was mixed with 1 ml of bio-rad dye and vortexed. The mixture was incubated at room temperature for 5 min. Absorbance was read at 595 nm using spectrophotometer. Absorbance values were converted to protein concentration by extrapolation from the standard curve.1 ml of bio-rad dye and I ml of buffer were used as blank (Bradford, 1976).

Assay of polygalacturonase activity

The reaction mixture contained 0.1 ml of the enzyme and 0.8 ml of citrus pectin. The mixture was incubated for 20 min at 40°C. After incubation, 1 ml of dinitrosalicylic acid (DNS) was added and the mixture heated at 90°C for 5 min to develop the color.

After cooling, the absorbance was read at 575 nm using a spectrophotometer. A blank of DNS and buffer was used to zero the spectrophotometer. Absorbance values were used to calculate the activity of the enzyme using the standard curve. One unit of enzyme activity was defined as the amount of enzyme that produced 1 µmole of D-galacturonic acid per minute at 40°C and pH 5.0 (Soares et al., 1999).

Ion-exchange chromatography (Fast Protein Liquid Chromatography, FPLC)

15 ml of the crude enzyme was loaded onto the column (15.5 cm x

1 cm). Fifty-six fractions of 5 ml each were collected at a flow rate of 3 ml per minute. Each fraction was assayed for activity and protein content. An elution profile was plotted to show active peaks.

Gel filtration (FPLC)

1 ml of the enzyme from Ion-exchange chromatography was gradually injected into the pre-equilibrated column. Filtration was carried out for 2½ h. Fifty-six fractions were collected and protein content and activity were determined. The fractions with high activity were pooled and used for characterization and electrophoresis.

Immobilization of polygalacturonase with sodium alginate

2.39 µg of partially purified enzyme was mixed with 4.0 ml of 3% sodium alginate solution in a ratio of 1:1. The beads were formed by dropping the polymer from a height of approximately 20 cm into an excess (100 ml) of stirred 0.2 M $CaCl_2$ solution with a syringe and needle at room temperature. The bead size which was controlled by the pump pressure of the hypodermic needle was 2.0 mm in diameter and a volume of 4.18 cm^3. The beads were left in the calcium chloride solution for 3 h to mature. The entrapped enzyme was used for characterization as previously described for the native enzyme.

Initial velocity studies of immobilized polygalacturonase

The effect of substrate concentration on the activity of immobilized polygalacturonase was determined by incubating the enzyme with 1, 3, 5, 7, 9, 10, 13 and 15 mg/ml of citrus pectin, respectively. The activity of the enzyme was assayed at each substrate concentration using the method of Miller (1959).

The apparent V_{max} and K_M of the immobilized polygalacturonase was determined using double reciprocal plot (Lineweaver and Burk, 1934).

Effect of pH change on the activity of immobilized polygalacturonase

The optimum pH of the immobilized polygalacturonase was determined using acetate buffer with a range of pH 3.5 - 5.5 by putting the enzyme beads in 0.05 M acetate buffer prepared at the various pH and assayed for activity as per the method of Miller (1959). A plot of activity versus pH was made to determine the optimum pH.

Effect of temperature change on the activity of immobilized polygalacturonase

The optimum temperature of immobilized polygalacturonase was determined by incubating the enzyme at 30 - 60°C at interval of 5°C for 20 min and assayed for activity as per the method of Miller (1959). A plot of temperature versus activity was made to obtain the optimum temperature of the enzyme as per the method of Stauffer and Etson, 1969).

Determination of pH stability of immobilized polygalacturonase

The enzyme was mixed with 0.05 M acetate buffer pH 3.5 - 5.5, phosphate buffer p H 6.0 - 7.5 and Tris-HCl buffer pH 7.5 - 9.0 and maintained at room temperature for 24 h. An aliquot of the enzyme was used to determine the residual activity at 40°C as per the method of Miller (1959). A plot of activity versus pH was constructed to determine pH stability.

Determination of temperature stability of immobilized polygalacturonase

The enzyme was incubated at 30 - 70°C at interval of 10°C per hour at pH 4.5. An aliquot (0.1 ml) of the enzyme was withdrawn and assayed for activity as per the method of Miller (1959). A plot of activity versus temperature was constructed to determine temperature stability.

Evaluation of catalytic activity of immobilized polygalacturonase per cycle

The first activity of the immobilized polygalacturonase was assayed at apparent optimum conditions (pH 4.5 and temperature of 40°C). The same enzyme was subjected to second and third catalytic cycles at 30 min interval to determine the catalytic stability of the enzyme.

RESULTS

The isolated polygalacturonase was precipitated at 70% ammonium sulphate saturation as shown in Figure 1 and purified nine fold with specific activity of 246 µmole/min/mg and a yield of 0.18% (Table 1).

The ion exchange elution profile of the enzyme on diethylaminoethyl (DEAE)- sepharose at 0 - 100% NaCl gradient revealed one active peak with highest activity of 1.1 µmole/min/mg as presented in Figure 2. Gel filtration profile of the enzyme gave one active peak at fractions 3 and 4. Both fractions contained protein and activity as presented in Figure 3. The apparent K_M and V_{max} values were 11.1 mg/ml and 1.65 µmole/min/mg, respectively, as presented in Figure 4.

The optimum pH and optimum temperature of the immobilized polygalacturonase was 4.5 and 40°C as presented in Figures 5 and 6, respectively.

As presented in Figure 7, the immobilized polygalacturonase was stable between pH 4.0 and 5.0. The enzyme was however, more stable at pH 5.0. The polygalacturonase was more stable at 50°C and least stable at 70°C as presented in Figure 8.

The immobilized polygalacturonase was used in three catalytic cycles at interval of 30 min. The percentage residual activity in relation to the first activity was evaluated to get the catalytic efficiency of the enzyme. As presented in Figure 9, the activity dropped from 100 to 34.56 and 14.81% in the second and third catalytic cycles, respectively. The half life of the enzyme was found to be 10 min.

DISCUSSION

The polygalacturonase was precipitated at 40 - 80% ammonium sulphate saturation with the highest activity of

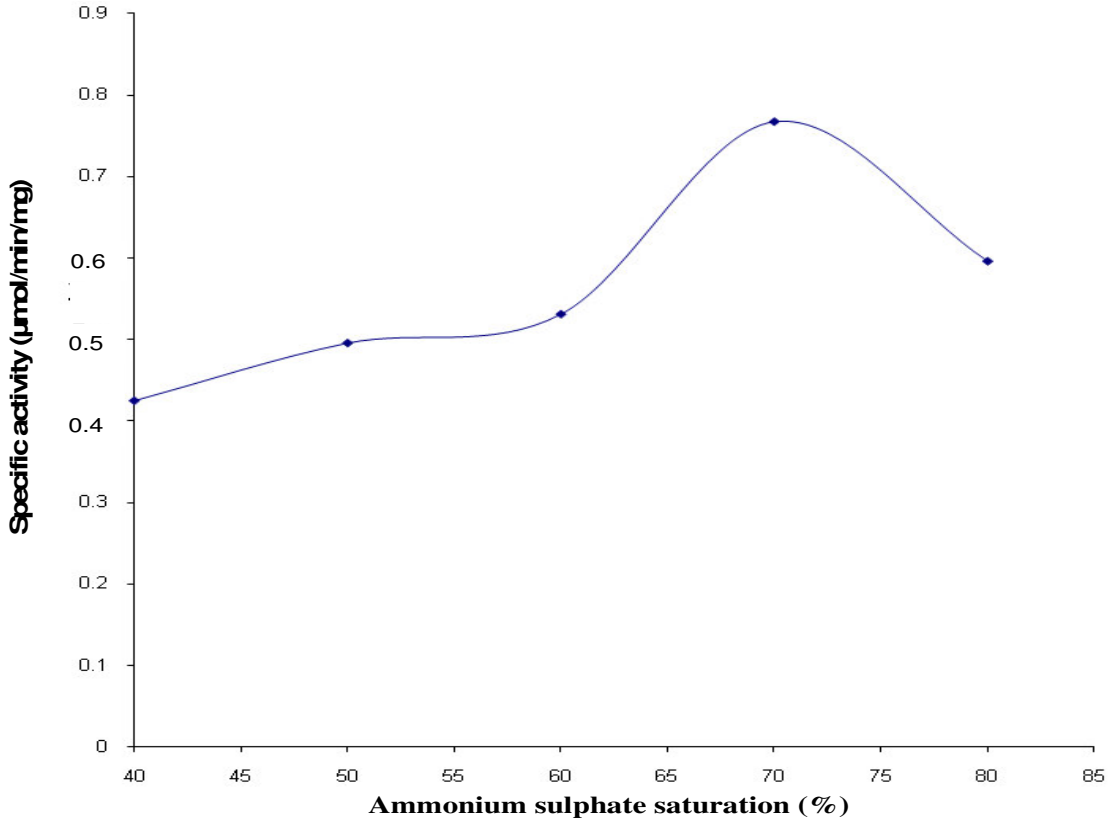

Figure 1. Ammonium sulphate precipitation profile of polygalacturonase from *Aspergillus niger* (SA6).

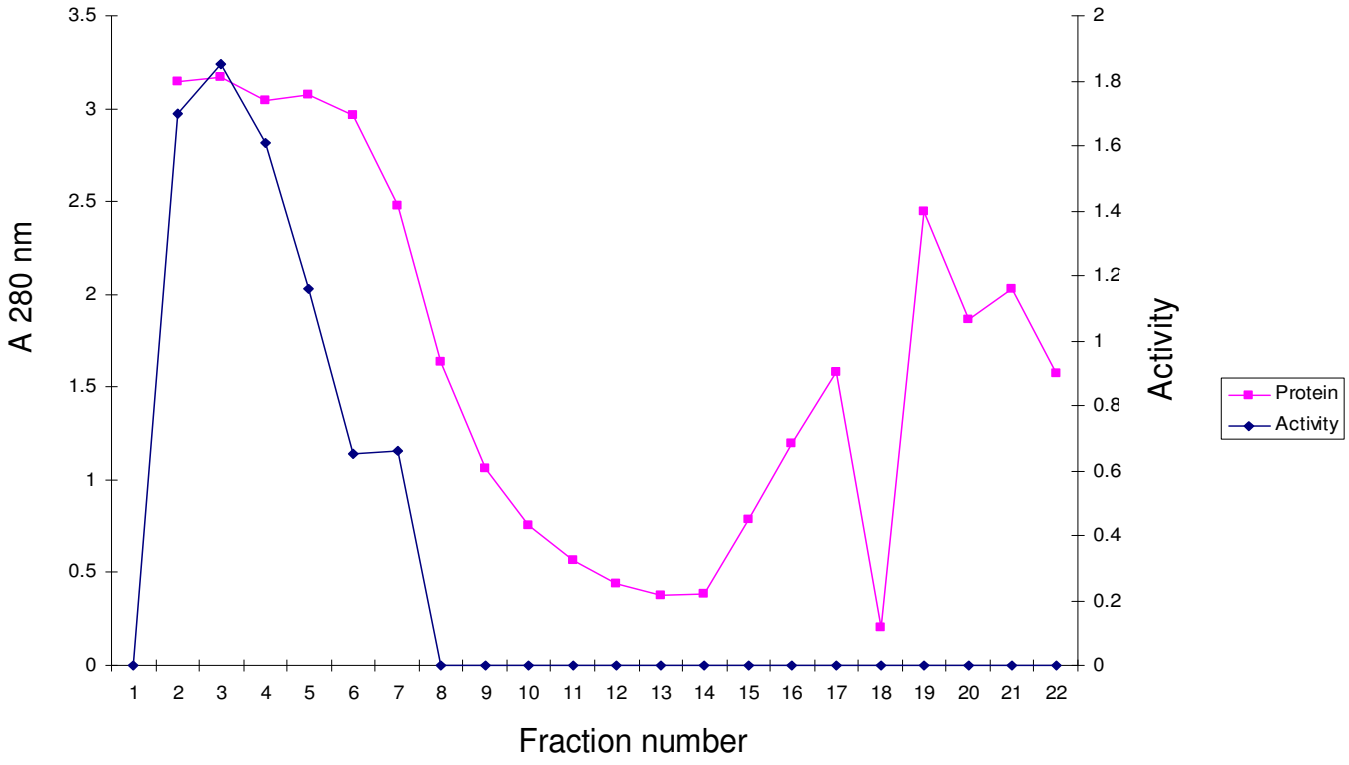

Figure 2. Ion-exchange profile of polygalacturonase from *Aspergillus niger* (SA6).

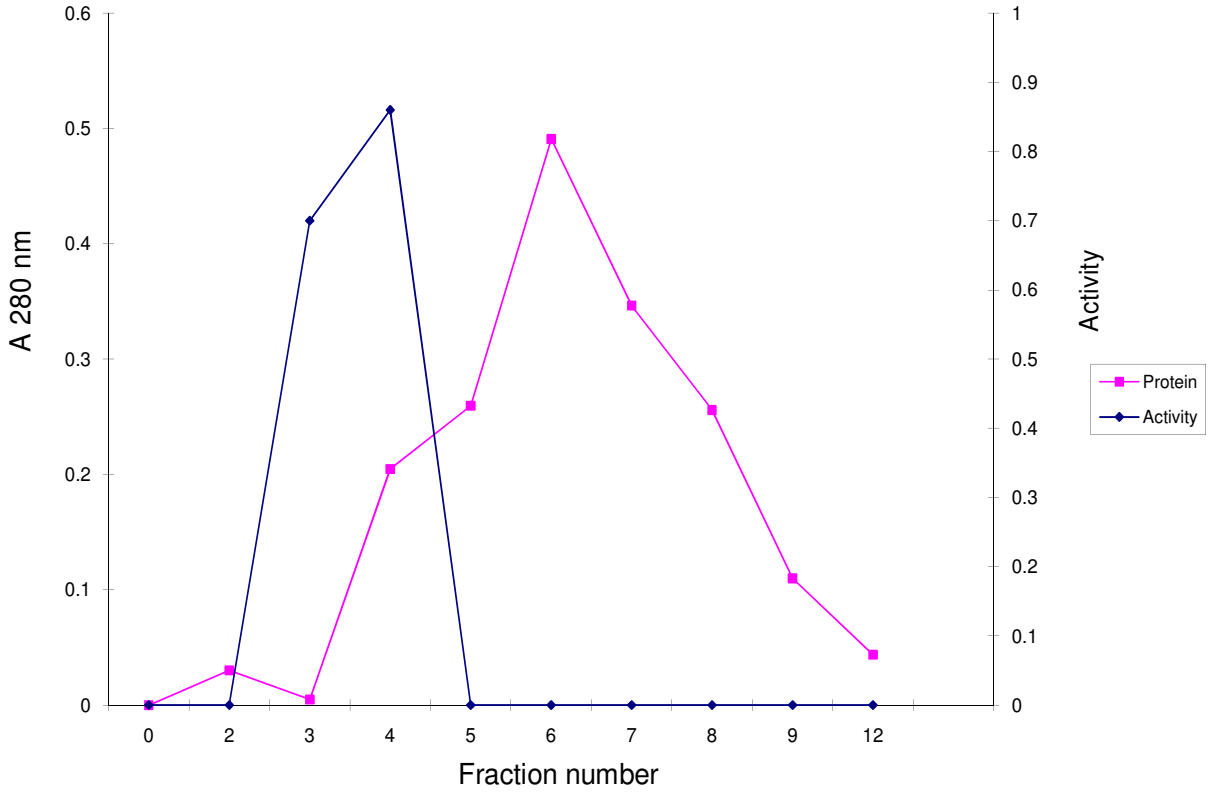

Figure 3. Gel filteration profile of polygalacturonase from *Aspergillus niger* (SA6).

0.78 µmole/min/mg obtained at 70% saturation.

Studies have shown that polygalacturonase can be precipitated between 0 - 90% of ammonium sulphate depending on the source of the enzyme (Bauman, 1981; Berger et al., 2000). This is consistent with the findings of this study. Ion-exchange chromatography profile of polygalacturonase on DEAE-sepharose showed that the enzyme was eluted in the flow through. This could be attributed to the fact that at pH 5, both the enzyme and the DEAE- sepharose carried the same, hence the protein did not bind the column and the enzyme was therefore eluted.

The gel filtration profile of the polygalacturonase revealed one active peak with the highest activity of 0.86 µmole/min/ml. The K_M and V_{max} of immobilized polygalacturonase calculated from Lineweaver-Burk plot were 11.1 mg/ml and 1.65 µmole/min/mg, respectively. When compared with the K_M and V_{max} of the native polygalacturonase, there was increase in both K_M and V_{max} after immobilization of the enzyme. The increase in K_M of the immobilized polygalacturonase may be due to inaccessibility of the substrate to the enzyme due to limitation in diffusion of substrate into the enzyme bead which is affected by the bead size, shape, pore size and enzyme loading per bead. The size and volume of the bead used in this study were 2.0 mm and 4.18 cm^3, respectively. The increase in the V_{max} as compared with that of the native enzyme may be attributed to increased stability of the enzyme after immobilization.

It was reported that the mass transfer limitation is manifested in an increase in the Michaelis Menten constant of the immobilized enzyme as compared with the K_M of the native enzyme. Thus, the value of apparent K_M is a good indicator of the extent of mass transfer resistance (Csiszar et al., 2001; Gupta et al., 1993; Stauffer and Etson, 1969). Pier et al. (2004) reported an immobilized polygalacturonase with a considerably high catalytic activity and higher apparent K_M value when compared with those of the native enzyme. Sartoglu et al. (2001) also reported a commercial immobilized pectinase with an increase in both K_M and V_{max} values after immobilization. This is consistent with the findings of this study. The optimum temperature of the immobilized enzyme remained 40°C. The immobilized enzyme is stable between pH 4.0 to 5.0 and more stable at 50°C when compared with the native enzyme which was more stable at 40°C. The broad pH stability which remained in acidic region could be an advantage in fruit processing because the natural environment of the enzyme is mildly acidic. The stability of the enzyme up to 50°C makes it less susceptible to thermal inactivation during fruit processing. The half life of the enzyme and the activity lost per minute on thermal storage were 10 min and 0.0213 µmole of D-galacturonic acid, respectively.

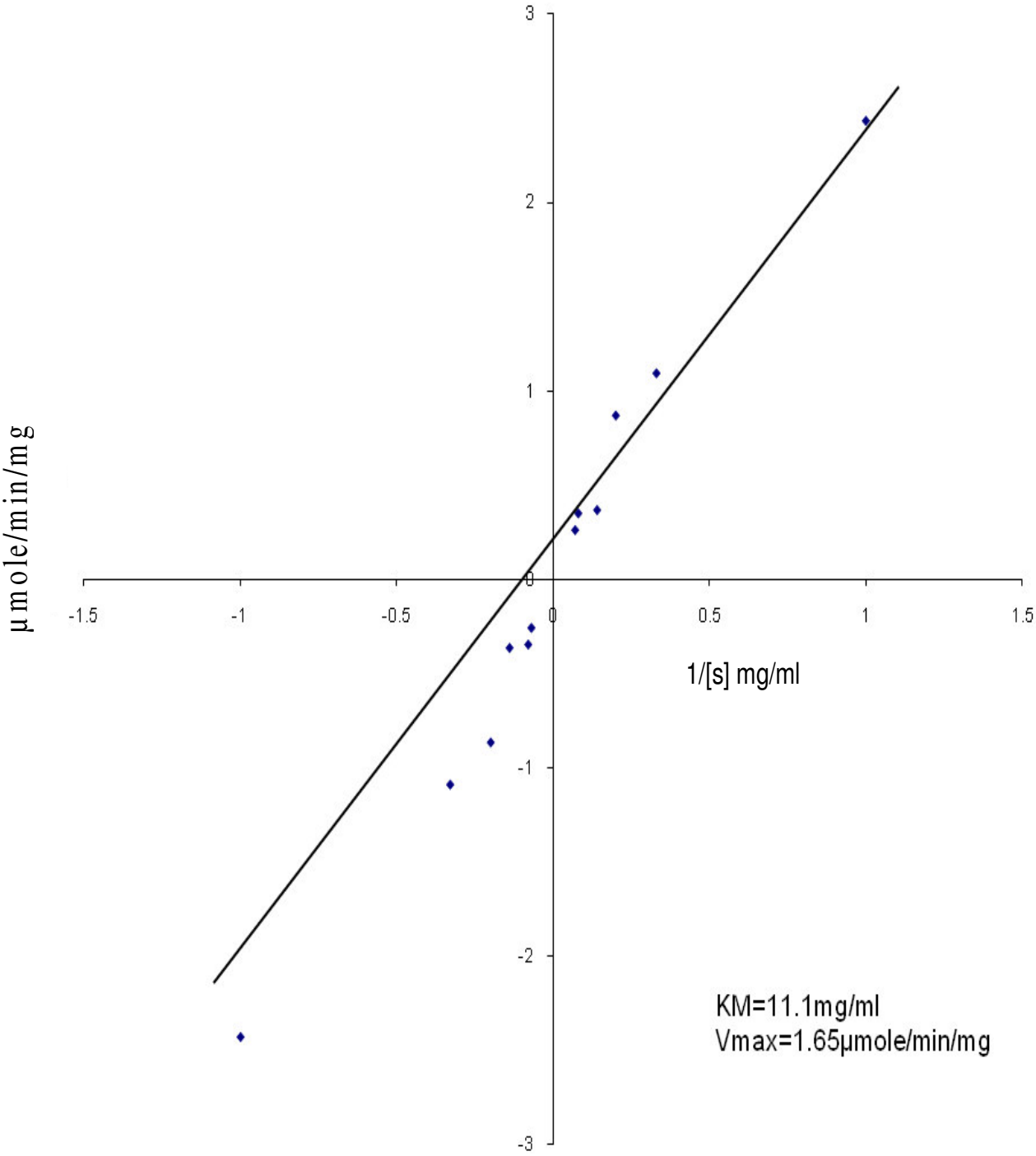

Figure 4. LineWeaver-Burk plot of immobilized polygalacturonase from *Aspergillus niger* (SA6).

The increase in pH stability could be due to the presence of positive and negative charges in the entrapped enzyme matrix. The increase temperature stability could be due to restriction of the enzyme and mass transfer resistance. It is generally recognized that kinetic parameters of immobilized enzymes are not true constants but apparent values and could change due to restriction of enzyme and substrate flow in and out of the bead.

In order to evaluate the efficiency of the immobilized polygalacturonase, it was subjected to three catalytic cycles. The second and third cycles reduced its catalytic activity to 34.56 and 14.81%, respectively. The reduction of activity may be due to heat inactivation and gradual

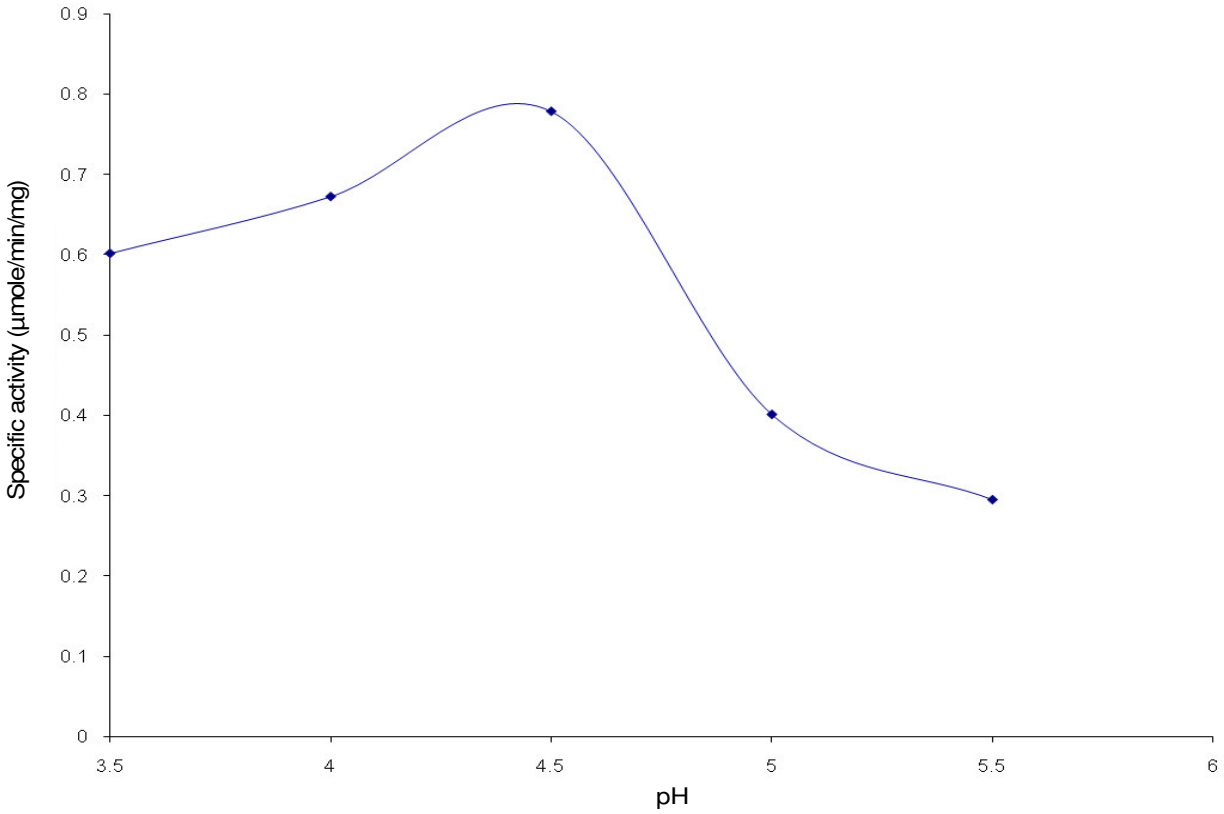

Figure 5. Effect of pH change on the activity of immobilized polygalacturonase from *Aspergillus niger* (SA6).

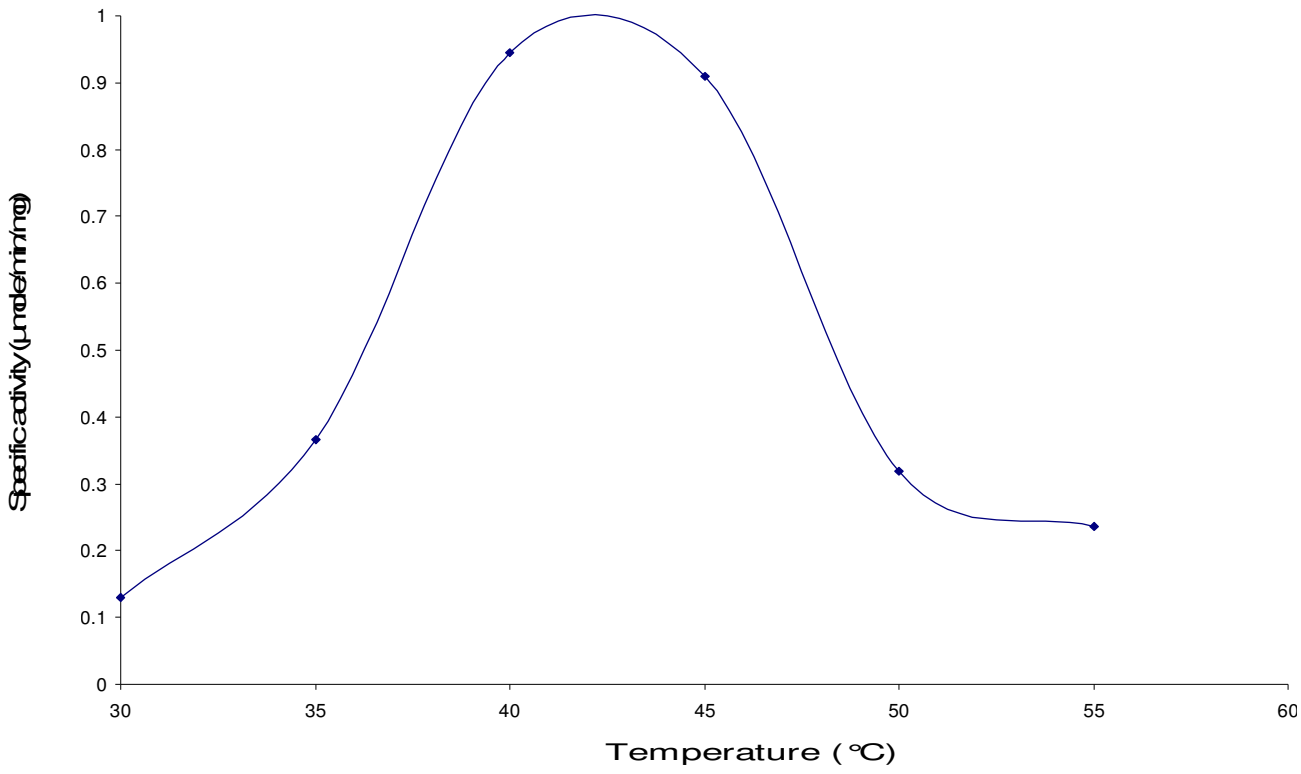

Figure 6. Effect of temperature change on the activity of immobilized polygalacturonase from *Aspergillus niger* (SA6).

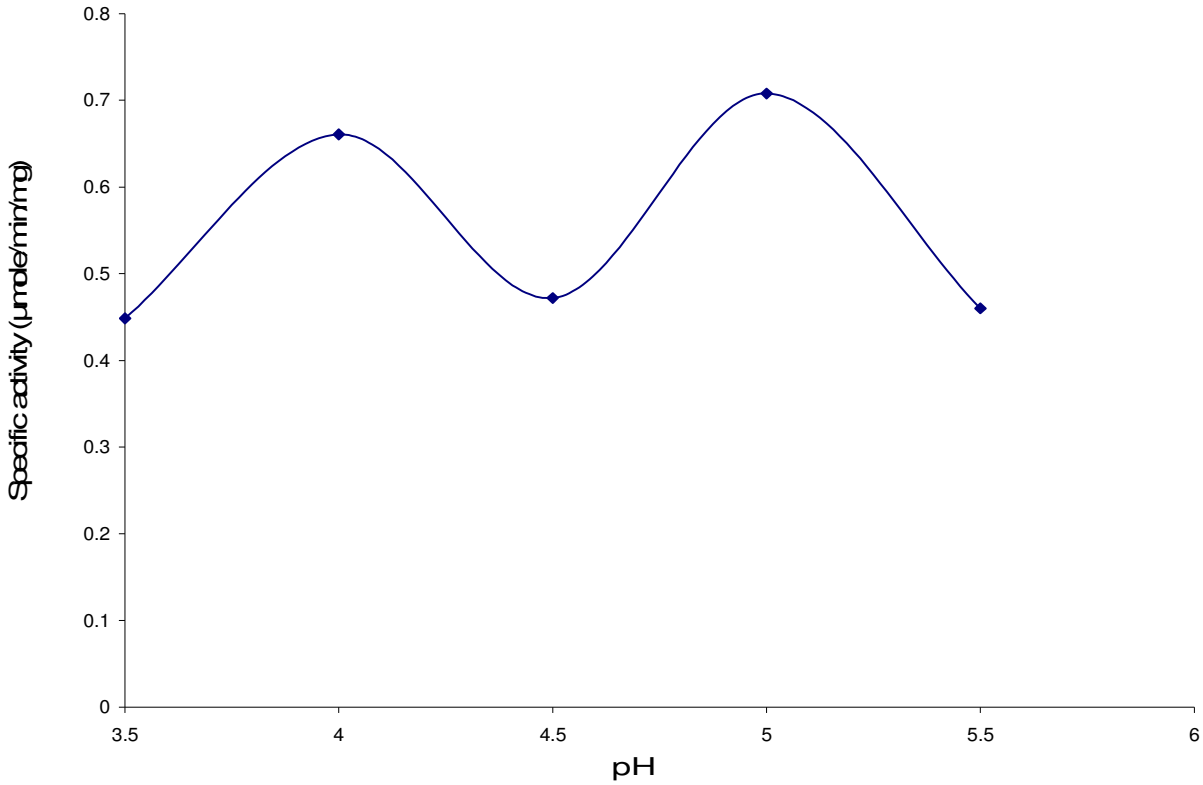

Figure 7. pH stability of immobilized polygalacturonase from *Aspergillus niger* (SA6).

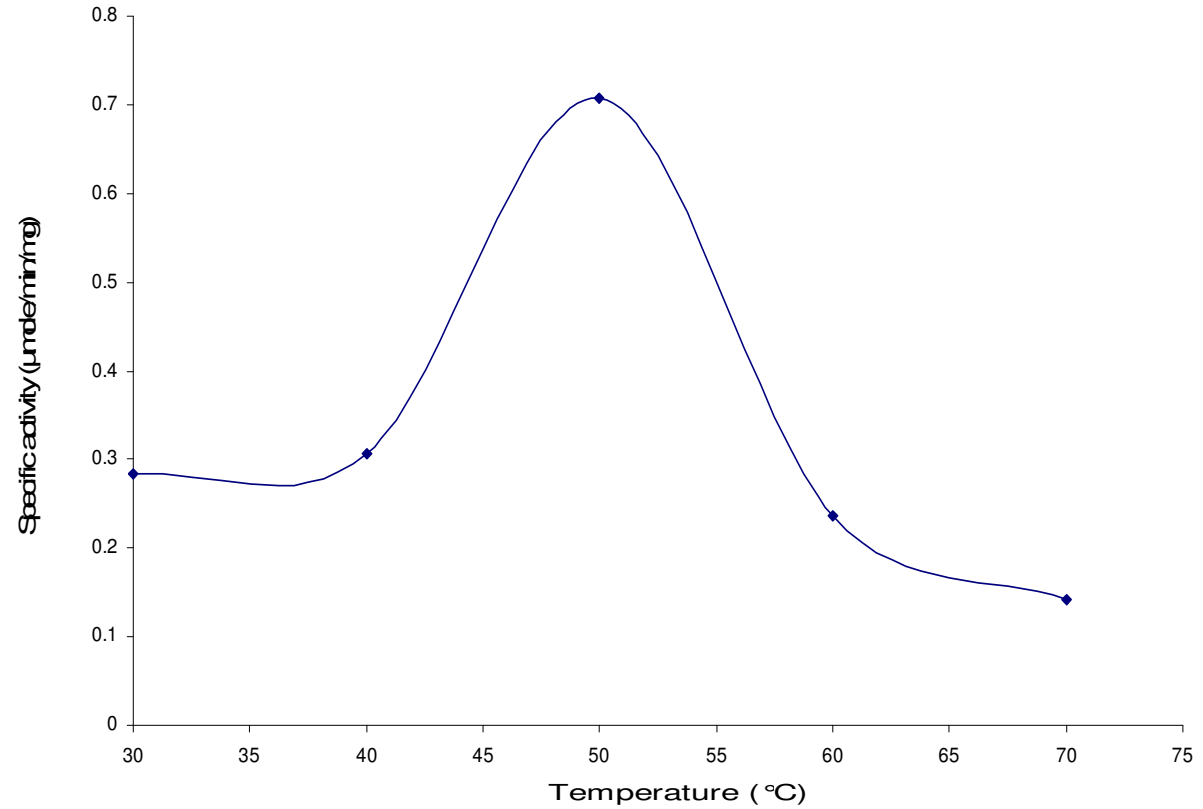

Figure 8. Temperature stability of immobilized polygalacturonase from *Aspergillus niger* (SA6).

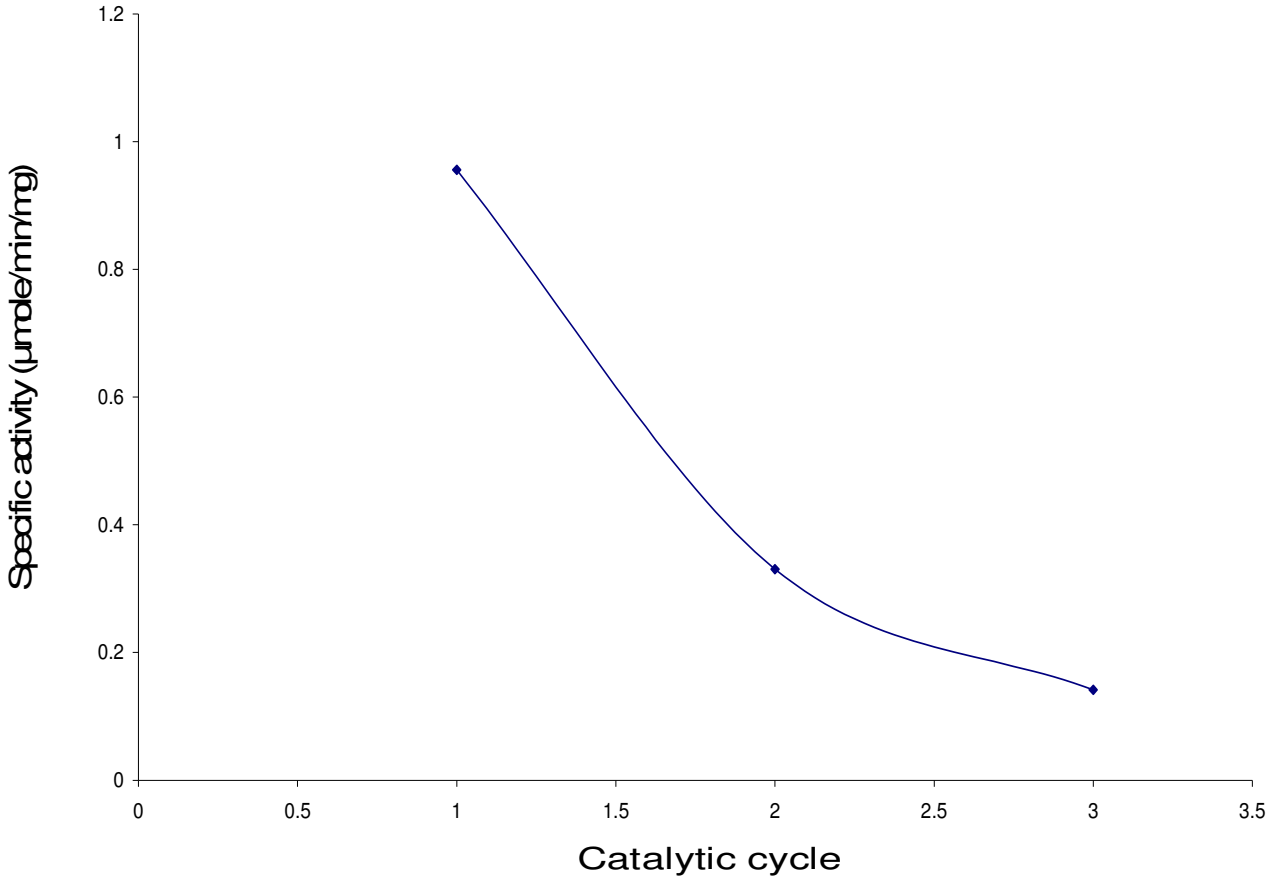

Figure 9. Activity of immobilized polygalacturonase from *Aspergillus niger* (SA6) per cycle.

loss of activity due to enzyme leakage. Entrapped enzymes usually suffer gradual loss of activity after several catalytic cycles due to leakage of the enzyme into the surrounding medium. Gupta et al. (1993) reported a pectin lyase that could be re-used through 4 cycles while Narsimha Rao et al. (2000) reported an endopolygalacturonase from *A. ustus* that retained 28% of its activity when immobilized and could be used through 10 cycles.

Conclusion

The enzyme has been partially purified, characterized and immobilized using sodium alginate. The immobilized polygalacturonase showed optimum activity at pH 4.5 and 40°C, respectively. It has been found to be stable over pH 4 - 5 (more stable at pH 5.0) and 50°C. The pH and temperature stability is an important feature of the enzyme that could be useful in fruit processing. The research has demonstrated that polygalacturonase can be used through at least three catalytic cycles with a half life of ten minutes. The data presented would help in designing immobilized polygalacturonase systems for use in fruit processing.

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