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Purification and Characterization of Protopectinase Produced by *Kluyveromyces marxianus*

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Abstract. The crude protopectinase preparation obtained from *Kluyveromyces marxianus* cultures was partially purified by fractional precipitation with ammonium sulphate. At 65% ammonium sulphate, the most active fractionation was obtained. Further purification by gel filtration on CM-Sephadix C-50 followed by ion exchange chromatography on Sephadix G-75 yielded 4 peaks of protopectinase components. The second peak was the major one containing most of the recovered protein and all the protopectinase activity.

The enzyme showed different hydrolytic activities on some protopectin sources. Characterization of the enzyme including, enzyme concentration, amount of substrate, pH and temperature of the reaction mixture was carried out. The enzyme was stable up to 50°C and at pH range between 4 and 7. The enzyme activity was responded differently to the tested metal ions and some inhibitors. The amino acids composition of the enzyme showed a high proportion of glycine and moderate amounts of glutamic acid, alanine and leucine but poor contents of proline, cysteine and tyrosine.

Keywords. Protopectinase, *Kluyveromyces marxianus*, Protopectin.

Introduction

The name pectin releasing enzymes or protopectinases is applied to a heterogeneous group of enzymes that solubilized pectin from protopectin, a water insoluble parental pectic substance present in plant tissues[1]. These enzymes have great commercial importance for various applications[2, 3]. Several protopectinases from different microorganisms (bacteria, yeast and filamentous fungi) have been purified and characterized[4]. Major impediments to the

exploitation of commercial enzymes are their yield, stability, specificity and the cost of production. New enzymes for use in commercial applications with desirable biochemical and physicochemical characteristics and low production cost have been the focus of much research[5].

In a previous work[6] the different fermentation conditions that lead to maximum bio-extraction of grapefruit waste pectin by *Kluyveromyces marxianus* and production of highly active protopectinase were optimized. The purpose of this study was to isolate and purify protopectinase from *Kluyveromyces marxianus*, and to characterize some properties of the isolated enzymes.

Material and Methods

Grapefruit Waste

The waste was obtained from the local market, dried in an oven at 60°C for constant weight. The dried waste was finely ground in a Wiley mill and passed through a 60 gauge mesh to give a homogenous powder for routine use.

Microorganism and Cultivation

Kluyveromyces marxianus 70343 DSM (Deutsche Samplung Von Microorganismen) was maintained on agar slants containing 2% glucose, 0.2% pectin and 0.1% yeast extract at pH 5[7]. For the seed culture, standard inoculum (4 ml yeast suspension / 100ml medium) of 24h old culture of the yeast was allowed to grow in a medium of 2% glucose, 0.4% peptone, and 0.2% yeast extract, pH 5 at 30°C for 24h under shaking conditions. The yeast was cultivated in 60ml aliquots/250ml Erlenmeyer flasks with the following medium: grapefruit waste 8%, glucose 1.0%, peptone 0.4%, pH 6, and inoculated with 4% inoculum size (85×108 yeast cells/ml), 24h old seed culture, incubated at 30°C for 18h under shaking conditions (200 shakes / min, amplitude 7cm). These conditions favored maximum pectin bioextraction and highest protopectinase activity[8].

Preparation and Precipitation of Crude Protopectinase

At the end of the incubation period, the residual grapefruit waste mixed with the yeast cells was separated by centrifugation at 4000 rpm for 20 min. The clear supernatant representing the crude enzyme extract was fractionally precipitate at different concentrations of ammonium sulphate in a sequential manner[9].

Purification of Protopectinase

1. The enzyme fraction precipitated at 65% ammonium sulphate was dialyzed against 0.03M acetate buffer, pH 5. The sample was applied to CM-Sephadex

C-50 column (30 × 2cm), previously equilibrated with the acetate buffer. Elution was then performed and 5ml fractions were collected at a flow rate of 20ml h^{-1}. The fractions that showed protopectinase activity were combined and lyophilized.

2. A sample from the protopectinase component resulting form the first column was re-chromatographed on Sephadek G-75 column (28 × 2cm) previously buffered (0.03M acetate buffer pH 5) and stepwise eluted in succession with 0.03M acetate buffer, pH 5(180ml); 0.06M acetate buffer, pH5 (200ml); 0.1M NaCl in 0.06M acetate buffer, pH 5 (250ml) and 0.5 NaCl in 0.06M acetate buffer, pH 5 (250 ml). Ten ml fractions were collected at a flow rate of 20ml h^{-1}. Fractions forming each protein component possessing protopectinase activity were combined, lyophilized and desalted. A unit of enzyme activity (U) was defined as the amount of enzyme required to produce one μmole of D-galacturonic acid min^{-1}, under the tested conditions.

Determination of Polygalacturonase Activity (Pectinase)

This was done by measuring the reduction in the viscosity of the buffered pectin solution[10]. A mixture of 1.5ml of the enzyme solution and 6ml of 1.0% pectin solution (in 0.02M acetate buffer pH 4.05) was incubated at 40°C for 20 min, then 5ml of the reaction mixture was pipetted into an Ostwald viscometer previously kept in a water bath at 40°C and the time of run was recorded. In control test, heated enzyme solution was employed. Polygalacturonase activity was calculated from the reduction in viscosity. One unit of polygalacturonase activity is the amount of enzyme producing 50% reduction in the viscosity of 0.5ml pectin solution[11].

Enzyme Characterization

This part of work deals with characterization of the pure enzyme obtained by pooling reactions of the second peak of protein recovered from the gel filtration column chromatography. Optimum enzyme concentration was carried out at 37°C for 30 min using 10mg grapefruit protopectin as substrate and the reaction pH was 5 using acetate buffer (0.02M). Optimum substrate concentration was carried out at 37°C for 30 min using acetate buffer at pH 5 and pure enzyme concentration of 87μg/reaction.

General Analytical Procedures

The protein content was determined by the method of Lowry[12] and modified by Tsuyosh and James[13], with crystalline serum albumin as a standard. Amino acid composition was determined using Beckman–Amino Acid Analyzer[14].

Enzyme activity was determined by measuring the amount of pectic substance liberated by carbazole sulfuric acid method as described by Wood and Kellogy[15]. The reaction mixture contains 10mg of protopectin, 40μmole of acetate buffer containing 50μmole of bovine serum albumin, pH 5 and 0.5ml of enzyme solution in a total volume of 2.5ml. The reaction mixture was incubated beforehand for 10 min at 37°C. The reaction started by the introduction of 0.5ml of enzyme solution and the mixture was kept for 30 min at 37°C. The reaction was stopped by cooling in an ice bath. The control blank was run using heat denaturated enzyme solution. After the reaction, the mixture was filtered on Toyo No. 2 filter paper (Toyo Roshi Co. Ltd., Tokyo, Japan). To a test tube containing 0.5ml of filtrate of the reaction mixture introduce 6ml of chilled 32 N H_2SO_4 solution, and then 0.5ml of 2% carbazole solution in ethanol. This step was done in an ice bath. The assay mixture was then heated at 80°C for 20 min and cooled to room temperature, after which the optical density at 525nm was determined. The pectin concentration was measured as D-galacturonic acid from the standard assay curve with D-galacturonic acid.

Optimum Ph and Temperature for Enzyme Activity

The activity was determined at optimum enzyme and substrate concentrations, at 37°C, at different pH values using acetate (pH 3-6) and phosphate (pH 7, 8) buffers (0.02M). The optimum temperature within the 25-70°C range was determined by incubation of the optimum conditions of enzyme and substrate levels, as well as, optimum pH value.

pH and Temperature Stability

Thermal stability of the purified protopectinase preparation was studied at pH 5 using acetate buffer. The enzyme solution was preheated at different temperatures (37°C to 70°C) for different periods. After each heat treatment, the substrate was added and the enzyme assay was carried out under the optimum conditions. For pH stability the enzyme (87μg/ml) in 0.02M acetate buffer (pH 3 to 6) or 0.02M phosphate buffer (pH 7 to 11) was incubated at 37°C for 30 min, and the remaining activities were determined after addition of the substrate under the optimum assay conditions.

Each treatment was carried out at least in triplicate and the recorded results represented the arithmetic mean.

Results and Discussion

Fractionatial Precipitation of Kluyveromyces marxianus Protopectinase

Partial purification of the crude protopectinase was fulfilled by fractional precipitation with ammonium sulphate. The total recovered protein of the 6

obtained fractions reached about 20.9% of the crude enzyme preparation (Table 1). Among all the obtained fractions the precipitated at 65% showed the highest protein recovery (8.4%). The same fraction showed a high recovery of the original protopectinase activity as well as the highest protopectinase/endopolygalacturonase ratio. The specific activity of the 65% ammonium sulphate fraction was 6.9 folds as that of the activity of the crude protopectinase enzyme present in the culture supernatant of *Kluyveromyces marxianus*. Similar good results concerning the precipitation of protopectinase or endo-polygalacturonase from yeast cultures were reported by many workers [7, 16].

Table 1. Fractional precipitation of the protopectinase enzyme present in the supernatant of *Kluyveromyces marxianus* cultures.

Ammonium sulphate saturation (%)	Total protein (mg)	Recovered protein (%)	PPA (μ/mg prot.)	Total activity (%)	Recovered activity (%)	EPGA (U/ml)	PAA / EPGA
CS	533	100	2.3	1226	100	1.32	4.7
25	13.1	2.5	0.0	0.0	0.0	1.05	0.0
35	16.1	2.8	3.5	52.5	4.3	0.63	8.4
50	19.6	3.7	4.6	72.5	5.9	1.06	8.6
65	44.6	8.4	15.8	705.1	57.5	1.47	9.6
75	11.8	2.2	2.1	24.8	2.0	1.08	2.1
85	7.3	1.4	0.0	0.0	0.0	0.66	0.0
Total	111.4	20.9	–	854.5	69.7	–	–

CS = cultures supernatant.
PPA = protopectinase activity.
sEPGA = Endopolygalacturonase activity.

Purification of Protopectinase by Column Chromatography

Further purification of the 65% ammonium sulphate fraction was carried out by subjecting this partially purified enzyme to a cation exchange chromatography column containing CM-Sephadex C-50. This process resoluted the protein into 3 peaks (Fig. 1). The major peak (No. 2) contained most of the recovered protein and protopectinase activity showing 41% protein recovery and 90 recovered activities. It was also noticed that the fractions forming this active peak did not show an endopolygalacturonase activity. This protopectinase active peak (16 folds purification) was gathered and rechromatographed on a Sephadex G-75 gel filtration column. The second column further fractionated the protein into 4 peaks (Fig. 2). The second peak was a major one containing

most of the recovered protein (52.4%) and all the protopectinase activity. The fractions forming this active peak were pooled and their activity showed 44% purification. Similar purification steps were also applied by others to obtain a highly pure protopectinase enzyme from other yeast strains[5, 7, 17-20].

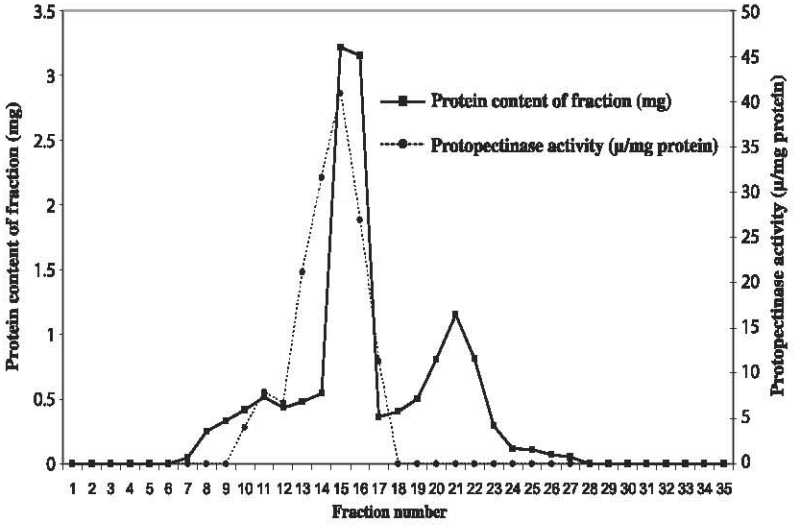

Fig. 1. Purification of protopectinase by ion exchange chromatography on CM-Sephadex C-50.

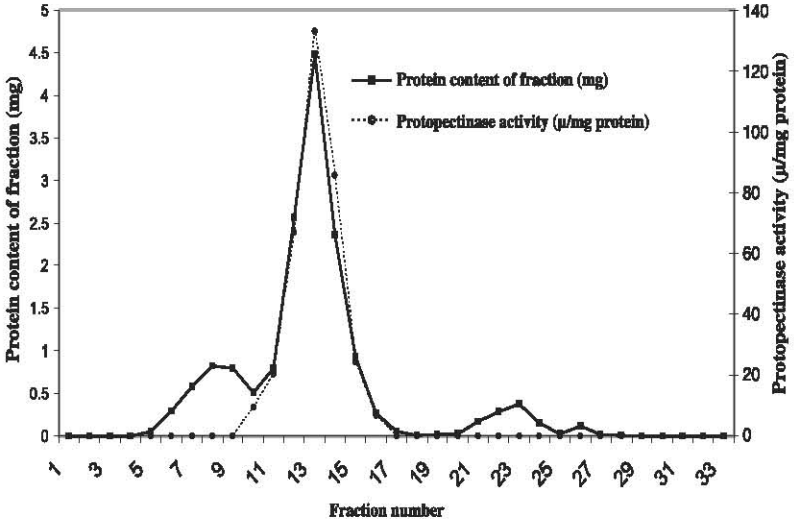

Fig. 2. Gel filteration on Sephadex G-75 of the protopectinase active peak obtained from the CM-Sephadex C-50 chromatography.

A summary of the purification stages of the protopectinase enzyme produced by *Kluyveromyces marxianus* cells from grapefruit waste is shown in Table 2.

Table 2. Purification of protopectinase enzyme produced in *Kluyveromyces marxianus* cultures.

Purification step	Total protein (mg)	Protopectinase activity (μ/mg prot.)	Fold purification	Total activity (U)	Yield (%)
1 – Cultures supernatant	2665	2.3	1.0	6129.5	100.0
2 – 65% AS fraction	223	15.8	6.9	3523.4	57.5
3 – CM-Sephadex C-50	45.6	36.9	16.0	1682.6	27.5
4 – Sephadex G-75	9.3	103.1	44.8	958.8	15.6

AS: Ammonium sulphate.

Characterization of the Purified Protopectinase

The first step was to elucidate the hydrolytic activity of the purified protopectinase on some protopectin sources. Results showed that the enzyme was active in hydrolyzing protopectin derived from mango peel, orange peel, lemon peel and beat pulp as well as grapefruit peel (Fig. 3). However, this enzyme showed different degrees of activity according to the type of substrate. It showed a low solubilizing action on protopectin of beet pulp. These may indicate that the protopectinase activity is dependent on the cell wall composition of the used substrate. Similar observations were reported by other workers[19, 22-23]. On the other hand, protopectinase derived from *Geotrichum* species was active on sugar beet pulp[24].

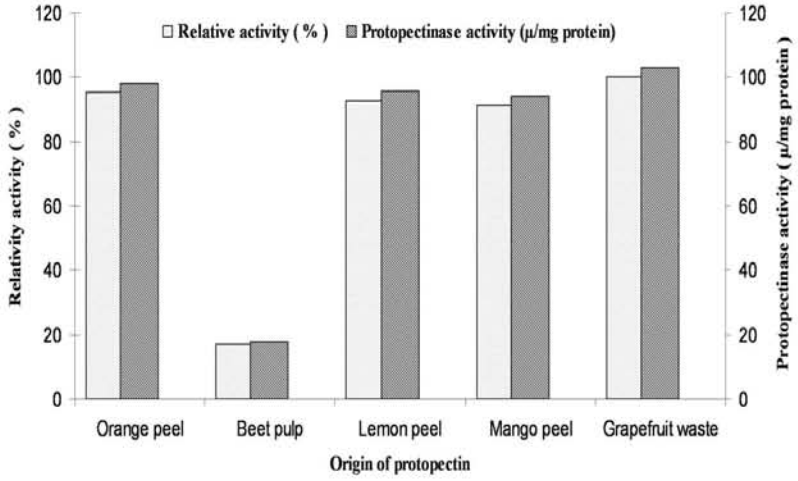

Fig. 3. Activity of pure protopectinase enzyme isolated from *Kluyveromyces marxianus* cultures on different protopectin substrates.

The results (Fig. 4) showed that a parallel relationship occurred between the pure enzyme concentration and its activity. The optimum enzyme protein concentration was 87µg/reaction mixture. It was also shown that optimum substrate (grapefruit protopectin) concentration was 20 mg / mixture (Fig. 5).

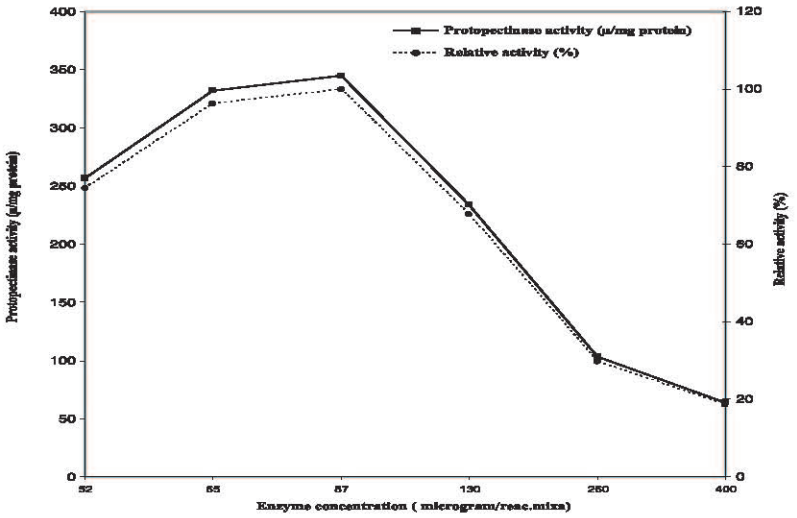

Fig. 4. Protopectinase activity of pure enzyme isolated from *Kluyveromyces marxianus* culture as influenced with enzyme concentration.

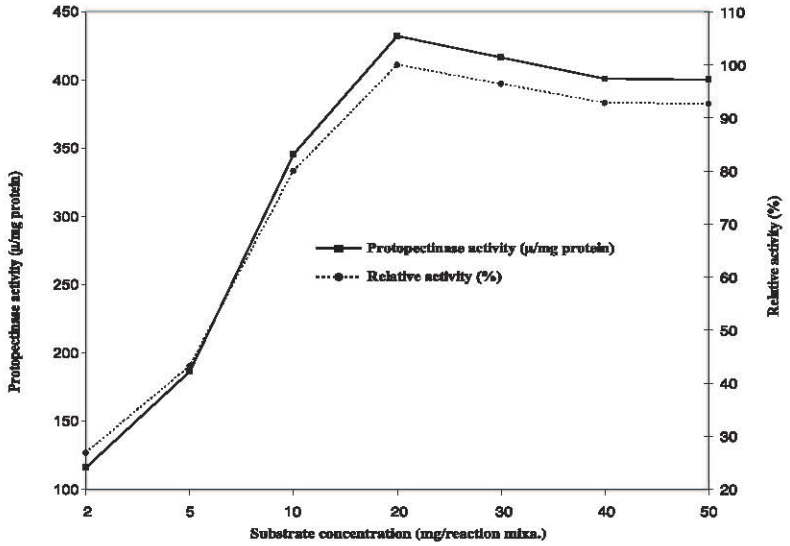

Fig. 5. Effect of substrate concentration on the protopectinase active of pure enzyme isolated from *Kluyveromyces marxianus* culture.

The data represented in Fig. 6 indicated that maximum activity of the pure protopectinase was achieved with the reaction mixture pH value equal to 5 using 0.02M acetate buffer. It showed also to be active in a pH range of 4 to 6. Similarly, maximal protopectinase activity at pH 4 to 5 of the enzyme isolated from *Galactomyces reesii* cultures was reported[17]. The optimum pH of a protopectinase activity, isolated by gel filtration in Sephadex C-50, derived from *Penicillium viridicatum* was 5[5]. The results of the present work also showed that the pH range of the protopectinase enzyme of *Kluyveromyces marxianus* is different than that produced by *Bacillus subtilis*, which was achieved at alkaline pH values[19, 25].

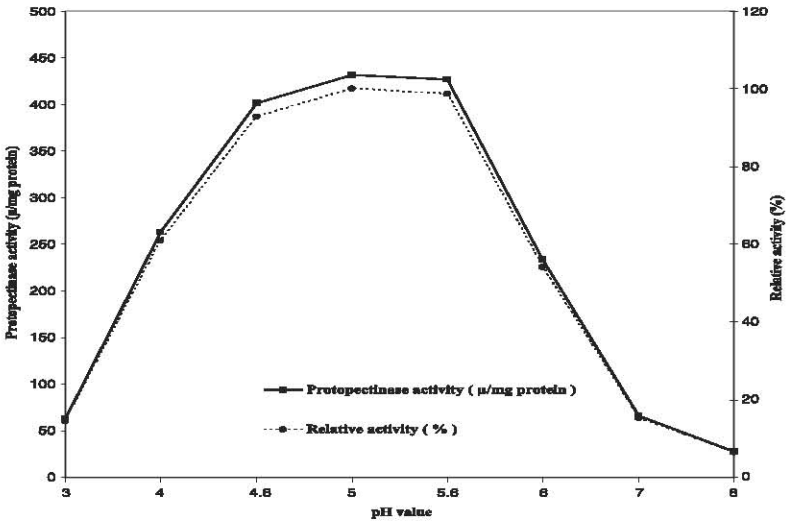

Fig. 6. Influence of pH of the reaction on the protopectinase activity of the pure enzyme isolated from *Kluyveromyces marxianus* cultures.

The activity of the pure protopectinase increased by increasing the reaction temperature (Fig. 7), reaching maximum value at an optimum temperature of 60°C. The enzyme showed also a good activity in a temperature range from 45 to 65°C. These values were slightly higher than those obtained from *Galactomyces reesii* which showed a maximum activity at 50°C[17], and than those of *Penicillium viridicatum*, obtained from agroindustrial waste, which showed a maximum activity at 50°C and 55°C[5]. The optimum temperature range of the obtained protopectinase was comparable to the values obtained for other protopectinase preparations[4,26].

The results (Fig. 8) showed that the enzyme was fairly stable up to 50°C since it retained 65% of its activity by exposure to this temperature for 30 min at pH 5. Longer time of exposure or higher temperatures resulted in the loss of a

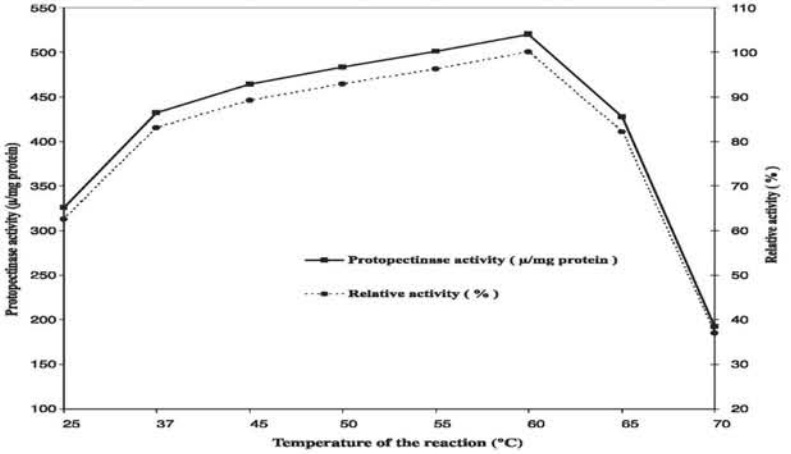

Fig. 7. Effect of temperature of the reaction on protopectinase active of pure enzyme.

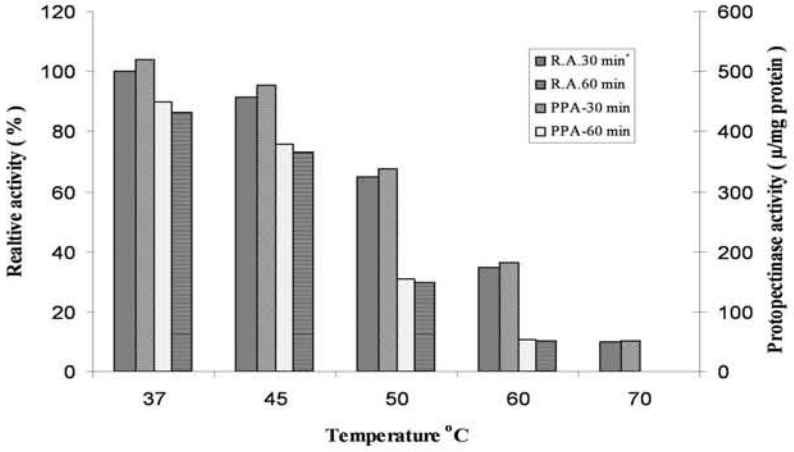

Fig. 8. Thermal stability of the pure protopectinase enzyme isolated from *Kluyveromyces marxianus* cultures.

major part of the enzyme activity and the enzyme lost 90% of its activity when exposed to 70°C for 30 minutes. This thermal stability profile obtained for *Kluyveromyces marxianus* protopectinase currently under investigation is nearly similar to the other protopectinases produced by different yeast[17, 26]. It is also similar to pectinases from bacteria and fungi[27-28, 5].

The presently investigated enzyme showed also to be stable in a pH range from 4 to 7 (Fig. 9). This pH stability is comparable to that obtained for a protopectinase preparation derived from *Galactomyces reesii*[29, 17]. While a protopectinase of *Penicillium viridicatum* was a stable at neutral pH[5].

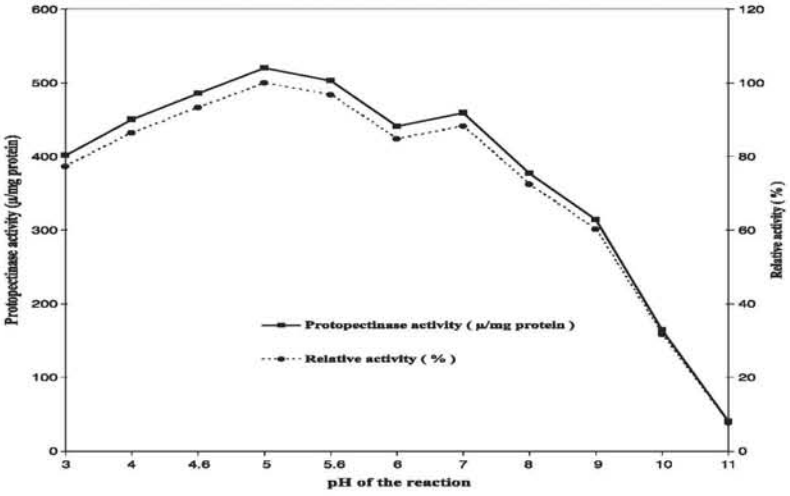

Fig. 9. pH-stability of the pure protopectinase enzyme isolated from *Kluyveromyces marxianus* cultures.

The effect of some ions and inhibitors on the activity of the purified enzyme (Fig. 10) indicated that Ca^{+2} and Mg^{+2} ions have a similar effect in maximum enzyme activity. On the other hand, the protopectinase enzyme lost most of its activity when treated with Cu^{+2}, Hg^{+2} or parachloromercuribenzoate. These results may indicate the presence of an active sulfhydryl group in the studied enzyme. Similar observations were made for other protopectinase enzymes[17,19, and 22]. They also reported that EDTA (a strong chelating agent) had only a slightly inhibitory effect on the protopectinase enzyme from yeast, a result similar to that obtained in the present work. The influence of trace elements of Fe, Zn, Mn, Co, Cu, and Mo on protopectinase activity of *Geotrichum klebahnii* indicated a critical role for their concentrations[30].

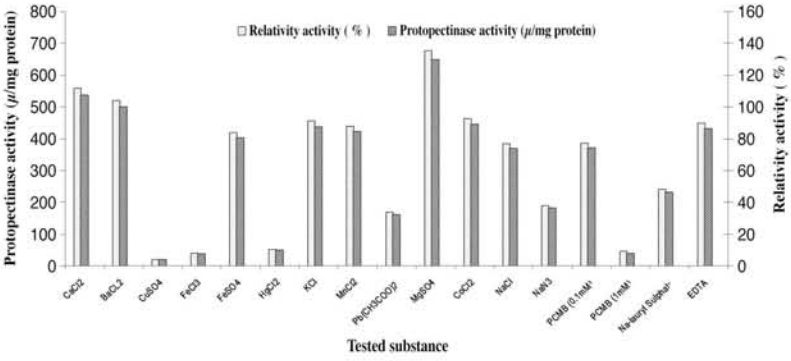

Fig. 10. Effect of some ions and inhibitors on protopectinase activity of pure protopectinase enzyme isolated form *Kluyveromyces marxianus* cultures.

The amino acid composition of the purified protopectinase of *Kluyveromyces marxianus* (Table 3) showed a high proportion of glycine. Glutamic acid, alanine and leucine were represented in a moderate amount, while proline, cystine and tyrosine were the lowest recorded amino acids. This protopectinase amino acid composition is comparable to the protopectinase composition obtained from *Trichosporon penicillatum* and *Kluyveromyces fragilis*[4, 7].

Table 3. Amino acid composition of the protopectinase enzyme isolated from *Kluyveromyces marxianus*.

Amino acid	Concentration mg AA/100 mg protein
Aspartic acid	5.78
Threonine	3.63
Serine	5.99
Glutamic acid	8.92
Proline	0.91
Glycine	27.43
Alanine	8.49
Cystine	0.31
Valine	3.82
Methionine	2.12
Isoleucine	3.76
Leucine	8.51
Tyrosine	0.21
Phenylalanine	2.97
Histidine	3.82
Lysine	4.40
Arginine	8.91

Conclusion

This investigation showed that the protopectinase produced by *Kluyveromyces marxianus*, from grapefruit waste as a substrate, is moderately stable and had a fairly high optimum temperature (60°C). It is also characterized by its higher activity on citrus peel, solubilizing partially these substrates to produce highly polymerized pectin. Accordingly, both the microbial culture or the enzyme could be effectively used in treating protopectin containing wastes, especially those resulting from the food industries and convert them to a valuable product.

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