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ISOLATION, PURIFICATION AND CHARACTERISATION OF PAPAYA PECTINESTERASE

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By

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LIST OF ABBREVIATIONS

cm	centimeter
g	gram
x *g*	times gravity
M	Molar
mM	millimolar
mg	milligram
min	minute
ml	milliliter
mmol	millimole
M.W.	molecular weight
var.	variety
v/v	volume per volume
v/w	volume per weight
w/v	weight per volume
w/w	weight per weight
μmole	micromole

Abstract of the Thesis Presented to the Senate of
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Pectinesterase (EC.3.1.1.11) is a pectic enzyme that can have a great impact in the fruit and vegetable processing technology because of its potential effect on the quality of the finished products. This project was designed to extract, purify and study the properties of the pectinesterase found in the papaya (*Carica papaya L.* var. Exotica) fruit. Studies carried out showed that the incubation time, pH and NaCl concentration influenced the extraction of the enzyme from the papaya fruit. A maximum activity of 7.0 μmole of carboxyl groups/min/ml (7.0 units/ml) was obtained when 2M NaCl solution (pH 8) and an incubation time of five hours were used. The procedure adopted for purification resulted in a 250-fold purification (784 units/mg protein) with a 45% recovery of the enzyme activity. The enzyme preparation was confirmed to be homogeneous by gel filtration and non-denaturing polyacrylamide gel electrophoresis and it has an apparent molecular weight of approximately 32,000 Daltons. The purified enzyme was further characterised as a

function of NaCl concentration, pH and temperature. Its kinetic properties were also studied. The activity was found to be linear up to 20 minutes with an enzyme concentration of up to 6.42 μg protein. Maximum activity was obtained using 0.25M NaCl solution, pH 8.0 and 65°C. The energy of activation of the enzyme was calculated to be 5690 cal mol^{-1}. A Q_{10} of 1.29 was obtained for the temperature range of 30 - 50°C at pH 7.0. The K_m value of the enzyme for citrus pectin as a substrate was 0.1125 mg/ml, corresponding to a V_{max} value of 730 μmole/min/mg protein. The Turnover number was calculated as 23360 mole/(mole.min). Inhibition studies showed that polygalacturonic acid acted as a competitive inhibitor. On the other hand, alginic acid exhibited a competitive-non-competitive type of inhibition while sucrose displayed an uncompetitive type of inhibition. The D values (time to inactivate 90% of the enzyme) at 55, 60, 65 and 70°C at pH 4.0 were estimated to be 112.14, 23.78, 8.33 and 1.71 minutes, respectively. Lower inactivation rates were observed for pH 7.5, with the D values ranging from 143.27 to 1.67 minutes for temperatures between 60 to 75°C. The Z values, which indicate the rise in temperature necessary to observe a 10 time faster rate of heat inactivation, were estimated to be 7.8°C and 8.38°C at pH 7.5 and pH 4.0, respectively. The inactivation energies and Q_{10} values were calculated as 256.9 kJ/mole and 15.59 at pH 4.0 and 284.76 kJ/mole and 19.21 at pH 7.5, respectively. pH stability studies showed that the enzyme was stable from pH 4-11 after exposure of the enzyme to these pH for 24 hours at 30°C. More than 85% of the activity was retained in all of these cases. However, at pH 1 and 12, the enzyme was unstable and it completely lost its activity after 24 hours of incubation at 30°C.

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PEMENCILAN, PENULINAN DAN PENCIRIAN PEKTINESTERASE BUAH BETIK

Oleh

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Pektinesterase (EC.3.1.1.11) adalah sejenis enzim yang penting dalam teknologi pemprosesan sayuran dan buah-buahan kerana ianya memberi kesan kepada kualiti produk akhir. Projek ini dijalankan untuk memencilkan, menulinkan dan mengkaji ciri-ciri pektinesterase yang didapati daripada buah betik (*Carica papaya L.* var. Exotica). Kajian yang telah dijalankan menunjukkan masa inkubasi, pH dan kepekatan NaCl mempengaruhi proses ekstraksi enzim daripada buah betik. Aktiviti yang maksimum iaitu 7.0 μmol kumpulan karboksil/min/ml (7 units/ml) diperolehi dengan menggunakan larutan NaCl 2M (pH 8) dan masa inkubasi 5 jam. Melalui kaedah penulinan yang dijalankan, didapati aktiviti pektinesterase (784 unit/mg protein) adalah menghampiri 250 ganda penulinan iaitu 45% pemulihan. Penyediaan enzim dipastikan dalam keadaan homogen melalui kaedah penurasan gel dan elektroforesis gel tidak nyahasli dan mempunyai berat molekul yang ketara menghampiri 32,000 Daltons. Kajian selanjutnya dijalankan bagi menentukan kesan

NaCl, pH dan suhu. Ciri-ciri kinetik juga dikaji. Didapati aktivitinya linear sehingga 20 minit pada kepekatan enzim 6.42 μg protein. Aktiviti maksimum didapati menggunakan 0.25M larutan NaCl, pH 8.0 pada suhu 65°C. Pengaktifan enzim ialah 5690 kal mol^{-1}. Q_{10} sebanyak 1.29 diperolehi pada julat suhu 30-50°C pada pH 7.0. Nilai K_m bagi enzim dengan pektin sitrus sebagai substrat adalah 0.1125 mg/ml bersamaan nilai V_{max} sebanyak 730 μmol/min/mg protein. Nombor pemulangan adalah 23360 mol/(mol.min). Kajian perencatan menunjukkan asid poligalakturonik bertindak sebagai perencat bersaing, sebaliknya asid alginik sebagai perencat bukan bersaing dan sukrosa pula perencat tidak bersaing. Nilai-nilai D (masa untuk menyahaktifkan 90 % enzim) pada 55, 60, 65 dan 70°C pada pH 4.0 dianggarkan selama 112.14, 23.78, 8.33 dan 1.71 minit masing-masing. Kadar penyahaktifan didapati lebih rendah pada pH 7.5 dengan nilai D adalah pada julat 143.27 hingga 1.67 minit pada julat suhu antara 60 hingga 75°C. Nilai-nilai Z iaitu nilai yang menunjukkan kenaikan suhu yang diperlukan untuk mendapatkan kadar 10 kali ganda penyahaktifan haba, dianggarkan menjadi 7.8°C dan 8.38°C pada pH 7.5 dan pH 4.0 masing-masing. Tenaga penyahaktifan dan nilai Q_{10} adalah 256.9 kJ/mol dan 15.59 pada pH 4.0 dan 284.76 kJ/mol 19.21 pada pH 7.5 masing-masing. Kajian kestabilan menunjukkan bahawa enzim stabil pada julat pH 4-11 selepas didedahkan kepada pH ini selama 24 jam pada suhu 30°C. Lebih daripada 85 % daripada aktiviti dapat dikekalkan dalam semua keadaan ini. Walaubagaimanapun, pada pH 1 dan 12, enzim ini tidak stabil dan hilang keaktifannya selepas diinkubat pada suhu 30°C selama 24 jam.

CHAPTER 1

INTRODUCTION

Pectinesterase (EC.3.1.1.11) is a pectic enzyme and is present in abundance in many fruits and vegetables (Versteeg, 1979; Voragen and Pilnik, 1989). The enzyme de-esterifies pectin, producing pectate and methanol (Rombouts *et al.*, 1979).

Pectinesterase has a great impact on the fruit and vegetable processing technology, because of its potential effect on the quality of the finished products (Pilnik and Rombouts, 1978a). Native pectinesterase in many fruits and vegetables may produce desirable and undesirable effects before, during or after processing (Versteeg, 1979). Endogenous pectinesterase can be used for protecting and improving the texture and firmness of several processed fruits and vegetables as well as in the extraction and clarification of fruit juices (Voragen and Pilnik, 1989). On the other hand, native pectinesterase is highly undesirable in the production of cloudy juices and it must be destroyed in order to maintain the desired stable cloud and viscosity (Underkofler, 1972). Gelation of papaya puree is also attributed to the enzymatic action of pectinesterase (Yamamoto and Inouye, 1963).

Pectinesterase has been purified and characterised from various plant sources such as from tomato (Lee and Macmillan, 1968; Nakagawa *et al.*, 1970a; Pressey and Avants, 1972), *Ficus awkeotsang* (Komae *et al.*, 1989; Lin *et al.*, 1989), apple (Castaldo *et al.*, 1989;

King, 1991), potato (Puri *et al.*, 1982), citrus (Manabe, 1973a; Evan and McHale, 1978; Versteeg *et al.*, 1978; Korner *et al.*, 1980; Seymour *et al.*, 1991) and it has been found that pectinesterases from different sources are different in terms of their molecular weight, specific activity, K_m values, stability to heat and pH and their response to pH, cation concentration and temperature for the enzyme activity. In fact pectinesterase from different varieties of the same fruit also differ in their properties (Pressey and Avants, 1972).

Reports available on pectinesterase from different varieties of papaya fruit have also shown a remarkable difference in their properties (Chang *et al.*, 1965; Lourenco and Catutani, 1984; Lim and Chung, 1989). Due to this variation in properties it seems necessary to purify and characterise the enzyme from other varieties of the papaya fruit which are commercially important. A Malaysian variety, exotica, also belongs to this group. This variety was introduced in 1987 by the Malaysian Agricultural Research and Development Institute (MARDI), Serdang. There has been much interest shown by entrepreneurs in planting the exotica on a large scale and 3,240 hectares have been grown in Peninsular Malaysia in 1988 (Ahmad, 1989).

A number of products can be processed from the papaya fruit and a large segment of the market of processed papaya is puree (Nordin and Adinan, 1989). The puree itself often serves as a source of raw material from which other papaya products are manufactured. Examples of products which may be made from papaya puree are juices, nectars, jams, jellies, syrups, toppings and dried fruit rolls or leathers

(Jagtiani *et al.*, 1988). The other products that can be processed from papaya are pickled products, dehydrated products, canned slices and frozen chunks (Nordin and Adinan, 1989).

Presently, there is no report available on the properties of the pectinesterase from the exotica variety. Therefore, the objectives of this study were:

1. To develop a procedure to extract the pectinesterase from the papaya (variety exotica) fruits.
2. To purify the enzyme and study its physical and chemical properties.
3. To characterise the purified enzyme and carry out kinetic studies.
4. To carry out stability studies on purified enzyme.

CHAPTER 2

LITERATURE REVIEW

Pectic Substances and Pectic Enzymes

Several literature reviews have been published on the pectic enzymes (Rombouts and Pilnik, 1972; Macmillan and Sheiman, 1974; Rexova-Benkova and Markovic, 1976; Rombouts and Pilnik, 1980; Pilnik and Rombouts, 1978b, 1981; Whitaker, 1972; Voragen and Pilnik, 1989). Rombouts and Pilnik (1978) also reviewed the importance of these enzymes in the fruit and vegetable juice processing technology and the literature on the industrial production and application of these enzymes was discussed by the same authors (Rombouts and Pilnik, 1980). The significance of the pectic enzymes in fruit and vegetable processing (Table 1) was reviewed by Pilnik (1982), Voragen and Pilnik (1989) and Pilnik and Voragen (1991). This chapter discusses current information available in the literature on various aspects of pectinesterases, specifically, and the other pectic enzymes in general.

Pectic enzymes are present in higher plants and are produced by some microorganisms (Pilnik and Voragen, 1991). They act on a variety of pectic substances which occur as structural polysaccharides in the middle lamella and the primary cell wall of higher plants (Pilnik and Rombouts, 1978b).

Pectic substances are complex heteropolysaccharides composed principally of α-1,4 linked galacturonic acid or its methyl ester (Figure 1).

Table 1.

Technological Roles and Applications of Pectic Enzymes (Adopted from Voragen and Pilnik, 1989)

Enzyme (s)	Technological roles/applications	
	Endogenous	Exogenous
PE	- Cloud stability in citrus juices - Pectin manufacture from citrus pomace - Distillates from fermented fruit pulps - Enzymatic maceration of fruits and vegetables - Self-clarification of lemon/lime and apple juices - Pressing characteristics of citrus pomace - Ca^{2+}-firming of fruits and vegetables	- Manufacture low-ester pectins with reduced Ca^{2+} sensitivity - Apple juice clarification
PG	- Softening during ripening (pears, peaches, avocado) - Texture loss of canned or bottled fruit products	- Citrus juice stabilisation - Maceration (concentrated nectar bases, baby food, vegetable juices) - Viscosity reduction of citrus concentrates - Manufacture of low viscosity pectins
PE + PG	- High viscosity tomato juices and pastes (hot break) - Low viscosity tomato juices and pastes (cold break) - Vegetable juices in combination with tomatoes	- Clarification (apple juice, pear juice, grape musts or wines) - Enzymic juice extraction (apples, soft fruit, stone fruit)

Cont.

Cont. Table 1

Enzyme (s)	Technological roles/applications	
	Endogenous	Exogenous
PE + PG		- Enzymic oil extraction (olives, palm fruit, coconut flesh - Depectinizing citrus pulp wash - Clouding agent from citrus peel - Recovery of oil from citrus peel
PL		- Maceration - Clarification; apple juice - Enzymic juice extraction
PAL		- Maceration
PG (+PE and/or PL)+(hemi)-cellulases		- Liquefaction; clear and cloudy juices (fruits + berries, tropical fruit, vegetables) - Cell wall destruction for isolation of interesting cell constituents - Liquefaction and saccarification of biomass

PE = Pectinesterase
PL = Pectinlyases
PG = Polygalacturonase
PAL = Pectatelyases

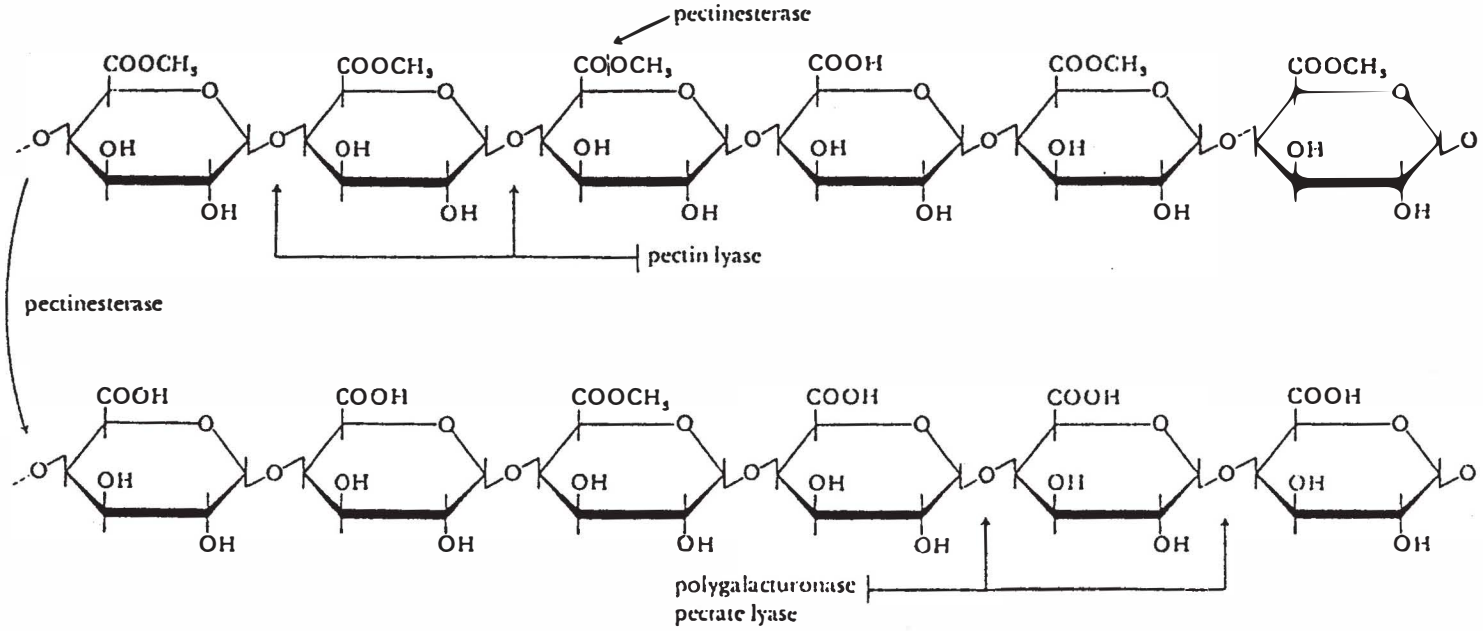

Figure 1. Fragment of a Pectin Molecule and Points of Attack of Pectic Enzymes.

(Adopted from Rombouts and Pilnik, 1980)

Partially methyl-esterified compound is known as pectin and the demethylated compound is known as pectic acid or polygalacturonic acid. The degree of esterification of a particular pectin depends upon its source and extraction procedure (Macmillan and Sheiman, 1974). Pectin also contains varying amounts of neutral carbohydrates such as L-rhamnose, L-arabinose and D-galactose along with traces of other neutral sugars (McCready and Gee, 1960). Various numbers of L-rhamnosylpyranose units are known to be linked through their carbon atoms C-2 and C-1 in the main galacturonan chain. More or less of the galacturonate residues may be acetylated at C-2 and C-3 positions depending on the plant source of the pectin (Rombouts and Pilnik, 1980).

Pectic enzymes are classified on the basis of their attack on the galacturonan part of the pectin molecule as shown in Figure 1. They are divided into two main groups, namely deesterifying enzymes (pectinesterase) and chain-splitting enzymes (depolymerases) (Pilnik and Voragen, 1991; Voragen and Pilnik, 1989; Pilnik, 1990). Pectinesterases (PE, EC.3.1.1.11) removes methoxyl groups from pectin transforming it into low methoxyl pectins or pectic acids (Figure 2). The depolymerases (Figure 3, Table 2) split the glycosidic linkages of their preferred substrates either by hydrolysis (hydrolases) or by ß-elimination (lyases). Polygalacturonases (PG; EC.3.2.1.15 and 3.2.1.67) split the glycosidic linkages next to free carboxyl groups by hydrolysis while pectate lyases (pectic acid lyases, PAL; EC.4.2.2.2 and 4.2.2.9) split glycosidic linkages by ß-elimination next to free carboxyl groups (Figure 3). They have an absolute requirement for calcium ions. Both endo- and exo-types of PGs and PALs are known. The endo-type (EC.3.2.1.15, EC.4.2.2.2) split the