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Kinetics of papaya pectinesterase

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Purified papaya (*Carica papaya* L. var. exotica) pectinesterase (EC 3.1.1.11) was investigated for its activity as a function of NaCl, pH and temperature, and determination of its kinetic parameters. The activity was linear up to 20 min with an enzyme concentration of up to 6·14 μg. Optimum activity was obtained with 0·25 M NaCl concentration and the optimum pH was found to be 8. The energy of activation of enzyme was 5690 cal mol^{-1}. A Q_{10} of 1·29 was observed in the temperature range of 30–50°C and the optimum temperature for the enzyme activity was 65°C. The K_m value for citrus pectin was 0·11 mg/ml, corresponding to a V_{max} value of 730 μmole/min/mg protein. The turnover number was calculated as 23 360 mole/(mole.min). Enzyme activity was found to be inhibited by the addition of polygalacturonic acid, alginic acid and sucrose in the reaction mixture and their K_i values were calculated as 0·019 mg/ml, 0·17 mg/ml and 29%, respectively. Polygalacturonic acid was found to act as a competitive inhibitor whereas alginic acid and sucrose showed a competitive–non-competitive and uncompetitive type of inhibition, respectively.

INTRODUCTION

Pectinesterase (PE; EC 3.1.1.11) is of prime importance to the food industry. It has a great impact on fruit and vegetable processing technology because of its potential effect on the quality of the finished products (Pilnik & Rombouts, 1978). It also plays a central role in the process of fruit softening during ripening; the control of its activity, through knowledge of dependence on such parameters as the temperature and pH, is of great practical importance in the food industry for protecting and improving the texture and firmness of several processed fruits and vegetables (Castaldo *et al.*, 1989). Preheating of potato tubers at 55°C for 15–20 min prior to freezing has been reported to improve the texture of frozen potatoes (Lester & Williams, 1969). According to Bartolome and Hoff (1972), pre-heating tubers at 55°C causes potato pectinesterase to act optimally on native potato pectin to produce free carboxyl groups which complex with divalent ions to form cross links between polyuronide chains and hence improve firmness.

Pectinesterase is widely distributed in numerous higher plants (Fayyaz, 1993). It has been isolated and characterized from different sources and pectinesterases from different sources show different kinetic properties; also pectinesterases from different varieties of the same fruit differ in their properties (Pressey & Avants, 1972). Reports that are available on pectinesterases from different varieties of papaya have also shown a remarkable difference in their properties (Chang *et al.*, 1965; Lourenco & Catutani, 1984; Lim & Chung, 1989), and it is therefore inadvisable to use these properties to optimize process conditions for varieties of the papaya fruit. A project was thus designed for the isolation, purification and characterization of pectinesterase from papaya (Malaysian variety exotica) fruit. In this part of the study several properties of the purified enzyme are described.

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MATERIALS AND METHODS

Citrus pectin (P-9135, galacturonic acid content 86%, methoxyl content 9·6%), polygalacturonic acid and alginic acid were bought from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used in this study were of analytical grade and most of them were obtained from Sigma.

Enzyme source

Papaya (*Carica papaya* L. var. exotica) pectinesterase, previously extracted and purified to homogeneity, was used in this study (Fayyaz *et al.*, 1993, 1994). The concentrated enzyme preparation had a specific activity of 780 μmole/min/mg protein (500 units per ml of enzyme solution). The enzyme was kept in 0·02 M, pH 7·5

sodium phosphate buffer solution containing 0·2 M NaCl and 0·02% sodium azide at 4°C.

Enzyme assay

The pectinesterase activity was determined by the method of Kertesz (1955), as described by Korner *et al.* (1980). Briefly, the method consisted of a titrimetric measurement of the rate of carboxyl group liberation from 1% pectin, 0·15 M NaCl solution at pH 7·0 and 30°C. The initial reaction velocity was measured by automatic titration of the liberated carboxyl groups with 0·02 M sodium hydroxide for 10 min in a Titralab Autotitrator model VIT 90/ABU 93/SAM 90 (Radiometer, Copenhagen, Denmark). Non-enzymatic de-esterification of pectin was determined in the presence of denatured pectinesterase and corrections were made to obtain the correct rate of the enzyme reaction. One pectinesterase unit is defined as the activity corresponding to the release of one micromole of carboxyl group per minute.

Effect of incubation time and enzyme concentration on enzyme activity

The activity of the enzyme as a function of incubation time and enzyme concentration was carried out under the standard assay conditions using 50 ml of 1% citrus pectin solution, pH 7·0, containing 0·15 M NaCl at 30°C temperature with different concentrations of enzyme and with different incubation times.

Effect of NaCl on enzyme activity

The effect of NaCl concentration on pectinesterase activity was tested in the range 0·05–0·5 M NaCl in the reaction mixture. Measurements were carried out under the standard assay conditions by using 1% citrus pectin dissolved in 50 ml of NaCl solution of varying concentrations at pH 7·0 and at 30°C.

Optimum pH for enzyme activity

The effect of pH on the enzyme activity was determined at different pH values ranging from 5 to 9. The enzyme assay was carried out under the standard assay conditions using 50 ml of 1% citrus pectin solution containing 0·15 M NaCl. All reactions were carried out for 10 min at varying pH values and at 30°C. Non-enzymatic de-esterification was determined at each pH value in the presence of denatured pectinesterase and corrections were made to obtain the rate of enzymatic reaction.

Effect of temperature on the enzyme activity

The effect of temperature on the activity of the enzyme was determined under the standard enzyme assay conditions with the exception of incubation temperature, which was varied from 5 to 80°C.

Determination of energy of activation and Q_{10} values

Energy of activation (E_a) was estimated from the slope of the Arrhenius plot obtained by plotting the L_n of the initial enzyme velocity against the reciprocal of the absolute temperature (Riet & Tramper, 1991). Initial velocities were determined at different temperatures under standard assay conditions. Q_{10} values were determined according to the method of Segel (1975).

Kinetics studies

All kinetic studies were performed under standard enzyme assay conditions. Michaelis constant (K_m) value, V_{max} value and K_i values were calculated from Lineweaver–Burk double reciprocal plots obtained by plotting $1/V$ versus $1/[S]$. The reaction mixture (50 ml, pH 7·0) contained 0·04–10 mg per ml of citrus pectin, 0·15 M sodium chloride and 2·57 μg (4 μl) of purified papaya pectinesterase. For the inhibition studies, measurements were carried out with and without the addition of 0·05 mg/ml, 0·1 mg/ml and 20% of polygalacturonic acid, alginic acid or sucrose, respectively, in the above-mentioned reaction mixture.

RESULTS AND DISCUSSION

Because a standard assay condition requires the reaction to be carried out under fixed conditions of pH, ionic strength and temperature, the experiments were performed at constant temperature (30°C), pH (7·0) and NaCl concentration (0·15 M) for 10 min throughout the study. An autotitrator enabled the course of reaction to be monitored automatically and continuously. But, after purification of the enzyme and before proceeding to the kinetic and stability studies, another experiment was designed to find the linearity of the reaction rate as a function of the incubation time as well as of the enzyme concentration because pectinesterase is directly dependent on both of these factors. The reaction was linear up to 20 min with the enzyme concentration up to 6·14 μg (10 μl of concentrated enzyme solution) (Fig. 1). The initial reaction velocity of micromoles carboxyl groups production was also proportional to the enzyme concentration up to 6·14 μg protein (10 μl of concentrated enzyme solution) under standard assay time of 10 min (Fig. 2).

After checking the linearity of the reaction as a function of the incubation time and enzyme concentration, the next experiment was designed to find the optimum conditions of NaCl, pH and temperature for the maximum enzyme activity. Experiments were each carried out under the standard assay conditions except for the parameter which was under study. In the first step, the effect of NaCl concentration on the purified enzyme activity was studied. Results showed (Fig. 3) that papaya (var. exotica) pectinesterase activity, like other plant pectinesterases, also depended on the NaCl concentration in the assay. The effectiveness of the salt was

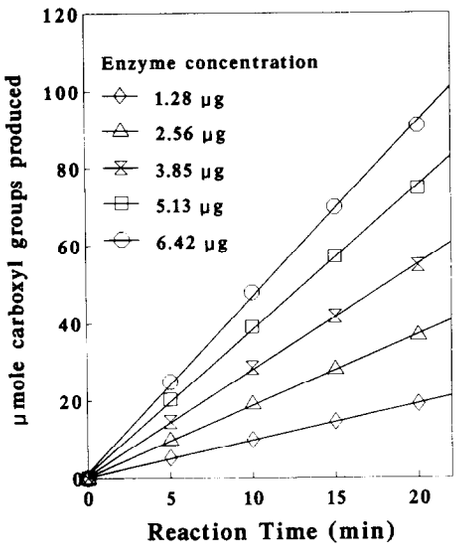

Fig. 1. Effect of time course on papaya pectinesterase activity.

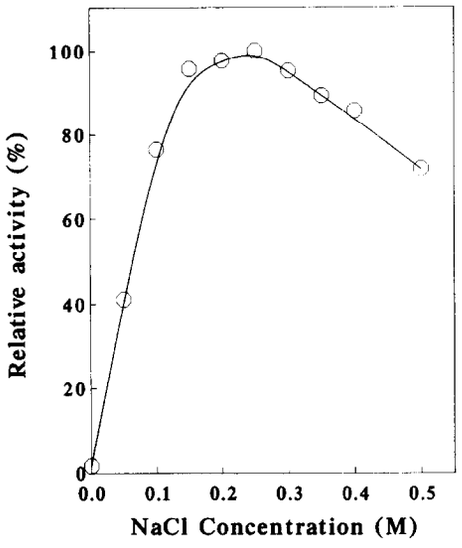

Fig. 3. Effect of sodium chloride concentration on papaya pectinesterase activity.

related to its concentration in the assay mixture and the activity increased with the concentration up to 0·25 M, but on raising NaCl level further the activity decreased. This optimum level of NaCl concentration obtained in this study is the same as that observed by Puri *et al.* (1982) for potato pectinesterase and is very close to the value of 0·2 M NaCl reported for papaya pectinesterase (Chang *et al.*, 1965; Lourenco & Catutani, 1984) and for *Ficus awkeotsang* pectinesterase (Lin *et al.*, 1989).

The stimulatory effect of NaCl on plant pectinesterases varies considerably and pectinesterases from different varieties of the same fruit have shown different optimum NaCl concentrations for their maximum activity (Lee & Macmillan, 1968; Nakagawa *et al.*, 1970; Pressey & Avants, 1972). According to this study, papaya pectinesterase possessed only 1·15% of the activity with 0·25 M NaCl as it did when assayed in the absence of the salt. The stimulatory effect of NaCl is quite high with 26, 49 and 61-fold increases in activity at 0·05, 0·1 and 0·15 M of NaCl concentration, respectively, as compared to the control (no NaCl added). The enzyme had approximately the same activity at NaCl concentrations of 0·15–3·0 M. As the concentrated enzyme solution contained 0·2 M NaCl, the contribution of NaCl from the enzyme solution may be considerable. However, since the volume of enzyme used in the study was very small relative to the volume of the substrate used, the contribution of the enzyme towards the overall NaCl concentration in the reaction mixture is negligible and can therefore be ignored.

The study of purified papaya pectinesterase activity as a function of pH shows (Fig. 4) that the activity increases rapidly from pH 5 to 7 reaching a maximum at pH 8·0 and remaining high over a range of alkaline pH. The optimum pH found is the same as the optimum reported by Versteeg *et al.* (1978) for navel orange pectinesterase (PE 2), and by Lourenco and Catutani (1984) for papaya (var. solo) pectinesterase.

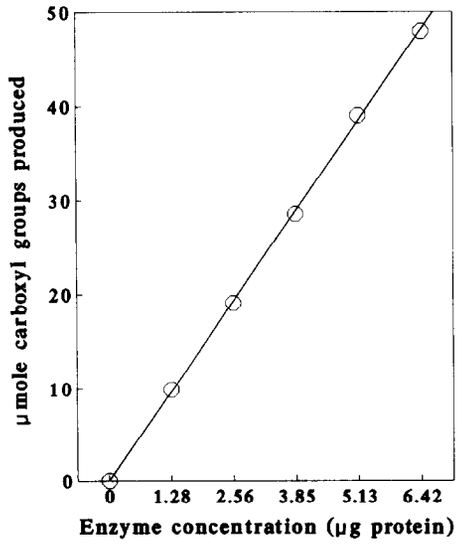

Fig. 2. Effect of enzyme concentration on papaya pectinesterase activity.

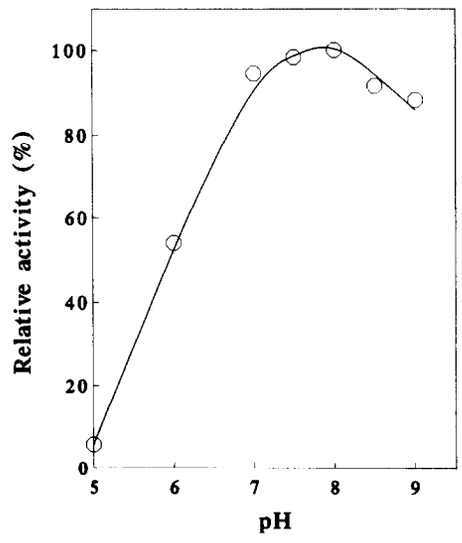

Fig. 4. Effect of pH on papaya pectinesterase activity.

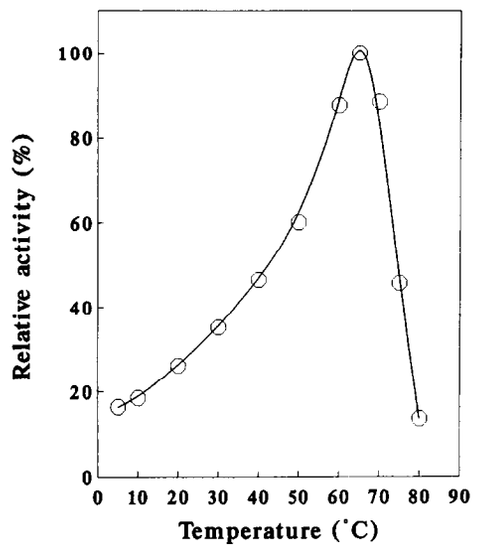

Fig. 5. Effect of temperature on papaya pectinesterase activity.

This value differs from that reported by Lee and Macmillan (1968) who found 8·5 to be the optimum pH for tomato (var. Heinz 135) pectinesterase and from Chang *et al.* (1965) who obtained a value of 7·5 for the papaya pectinesterase. According to Rillo *et al.* (1992), pectinesterase from mandarin orange fruit also exhibits a maximum activity at pH 9. The enzyme activity exhibited a strict pH-dependence; in fact, one unit of pH variation from 7 to 6 and 6 to 5 produced a 40% and a 48% decrease in enzyme activity, respectively. Measurements of pectinesterase activity above pH 8·0 were not reliable due to the extensive alkaline saponification of pectin (Rexova-Benkova & Markovic, 1976; Korner *et al.*, 1980).

Papaya (var. exotica) pectinesterase shows an optimum reaction temperature at 65°C (Fig. 5). Over the temperature range (5–80°C) studied, the rate of de-esterification appeared to increase from 5 to 65°C, reached maximum at 65°C and declined after 70°C. A six-fold increase in the reaction rate was observed when the temperature was raised from 5 to 65°C. It is interesting that the initial rate decreased sharply after 70°C. Similar values for temperature optimum (60°C) have been reported for orange pectinesterase (Korner *et al.*, 1980), papaya (var. solo) pectinesterase (Lourenco & Catutani, 1984) and for bramley apple pectinesterase (King, 1990). At the optimum temperature of 65°C, the enzyme under study showed a 2·78-fold increase in activity as compared to that under the standard assay temperature of 30°C. On the other hand, Lourenco and Catutani (1984) found an approximately six-fold increase for papaya (var. solo) pectinesterase when the temperature was raised from 30 to 60°C. Another study on papaya pectinesterase (the variety is unknown) gave a value of 35°C for its optimum temperature (Lim & Chung, 1989). From these results, it would seem that all of the pectinesterases from different varieties of the papaya are different in their properties.

According to Lee and Wiley (1970), activation energy may be regarded as the amount of energy needed to place the substrate molecule in a reaction state. If E_a is large, the rate of reaction will increase rapidly for a fixed increase in temperature. The substrate molecules must first absorb the amount of E_a; they can then react and be converted to products. The activation energy of the enzyme reaction was calculated from the slope of the Arrhenius plot, obtained by plotting the L_n of the initial enzyme velocity against the reciprocal of reaction absolute temperature (Fig. 6). The activation energy (E_a) of papaya pectinesterase was calculated to be 5690 cal mol^{-1} from 20 to 60°C (Fig. 7). These results are in agreement with the values of 5600, 5740, 5800 and 6200 cal mol^{-1} which have been reported for orange pectinesterase 1 and 2 (Korner *et al.*, 1980), apple pectinesterase (Lee & Wiley, 1970) and potato pectinesterase (Puri *et al.*, 1982). However, a considerably higher E_a value of 7644 cal mol^{-1} was reported for a crude preparation of pectinesterase isolated from Bramley apple waste (King, 1991).

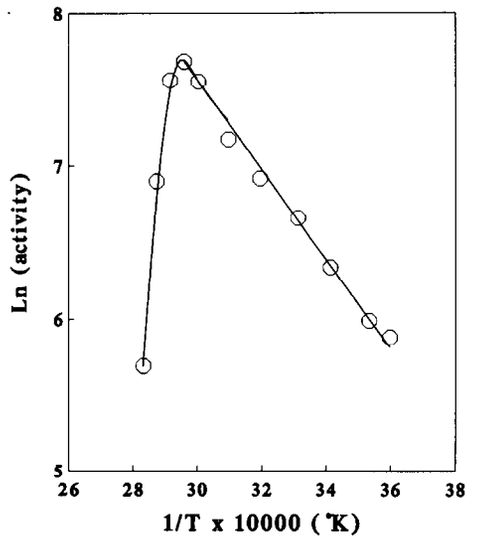

Fig. 6. Arrhenius plot for papaya pectinesterase.

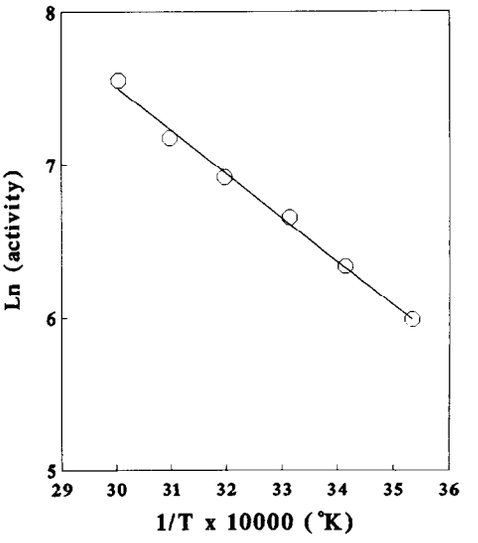

Fig. 7. Arrhenius plot for the reaction of papaya pectinesterase.

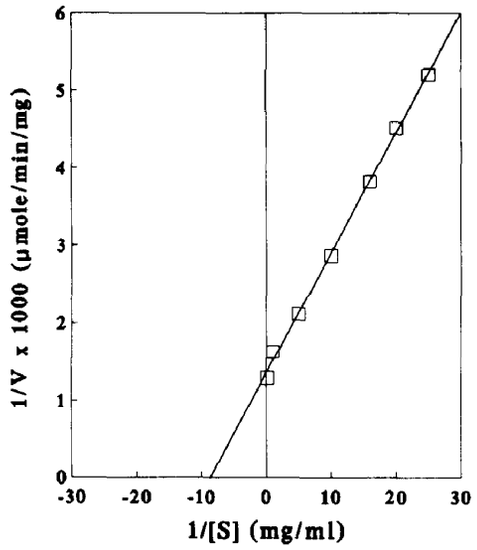

Fig. 8. Lineweaver–Burk plot of papaya pectinesterase activity as a function of substrate concentration.

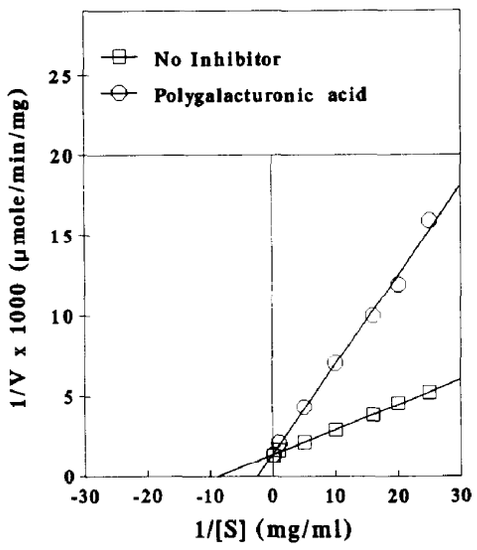

Fig. 9. Lineweaver–Burk plot of papaya pectinesterase activity with polygalacturonic acid inhibitor.

Nakagawa *et al.* (1970) have also reported higher E_a values (7800–9100 cal mol^{-1}) with the breaks in the Arrhenius plot for tomato pectinesterase.

A Q_{10} of 1·29 was observed in the temperature range of 30–50°C. This value is quite close to the values 1·33, 1·34 and 1·35 reported for tomato pectinesterase (Puri *et al.*, 1982), cucumber pectinesterase (Bell *et al.*, 1951) and pea pectinesterase (Collins, 1970), respectively.

The activity of the papaya pectinesterase as a function of pectin concentration was also investigated. The double reciprocal plot gave a K_m value of 0·11 mg/ml for citrus pectin (Fig. 8). All kinetic parameters are the means of at least two determinations and are reproducible. The K_m being reported here for papaya (var. exotica) is almost the same as that reported by Lourenco and Catutani (1984) for papaya (var. solo) pectinesterase (0·12 mg/ml) and it is close to the values of 0·083, 0·274, 0·29 and 0·3 mg/ml which have been reported for navel orange pectinesterase 1 (Versteeg *et al.*, 1978), marsh white grape fruit thermolabile pectinesterase (Seymour *et al.*, 1991), orange pectinesterase 1 (Korner *et al.*, 1980) and papaya pectinesterase (Chang *et al.*, 1965), respectively. The values for K_m vary widely from 0·0046 to 2·3 mg/ml for plant pectinesterases and vary even within the different varieties of the same fruit (Fayyaz, 1993). This difference may be related to the degree of purity of the enzyme and substrate but it seems that these differences are most probably due to the varietal differences of the fruit because these enzymes also differ in other properties such as response to temperature, pH and NaCl concentrations for their optimum activity.

Reaction velocity was determined under standard assay conditions and a double reciprocal plot gave a V_{max} of 730 µmole/min/mg (Fig. 8). This V_{max} value reported here for papaya (var. exotica) pectinesterase is almost the same as the value of 724 µmole/min/mg which has been reported for marsh white grape fruit thermolabile pectinesterase by Seymour *et al.* (1991), and very close to the values of 590, 564 and 561 µmole/min/mg reported for marsh white grape fruit thermostable pectinesterase (Seymour *et al.*, 1991) and navel orange pectinesterase 1 and 2 (Versteeg *et al.*, 1978), respectively. After knowing the V_{max} value for pectinesterase under study, the turnover number was calculated to be 23360 mole/(mole.min). This value of 23 360 mole/(mole.min) is very close to the values of 20400, 20280, 26354 and 30621 mole/(mole.min) which have been reported for navel orange pectinesterases PE 1 and PE 2 (Versteeg *et al.*, 1978) and marsh white grape fruit thermolabile and thermostable pectinesterases (Seymour *et al.*, 1991), respectively.

Inhibitors are substances which tend to decrease the rate of an enzyme-catalyzed reaction. In this study the effect of a few inhibitors such as polygalacturonic acid, alginic acid, galacturonic acid and sucrose were examined on papaya (var. exotica) pectinesterase. Works of earlier workers have shown that pectinesterase is inhibited by polygalacturonic acid and it acts as a competitive inhibitor (Lee & Macmillan, 1968; Versteeg *et al.*, 1978; Lourenco & Catutani, 1984). In this study pectinesterase was also found to be inhibited by the addition of polygalacturonic acid to the reaction mixture. The initial rates of de-esterification of the pectin were measured for various pectin concentrations in the absence and presence of polygalacturonic acid (Fig. 9). Polygalacturonic acid was also found to act as a competitive inhibitor of papaya (var. exotica) pectinesterase and the K_i value was calculated to be 0·019 mg/ml. This K_i value is in the range of values 0·0016, 0·07 and 0·416 mg/ml, which have been reported for navel orange pectinesterase 2 (Versteeg *et al.*, 1978), papaya (var. solo) pectinesterase (Lourenco & Catutani, 1984) and for navel orange pectinesterase 1 (Versteeg *et al.*, 1978), respectively. Even though the K_m value (0·11 mg/ml) for the papaya (var. exotica) pectinesterase is very close to the value for pectinesterase from another variety of papaya (var. solo) (0·12 mg/ml), their K_i values for

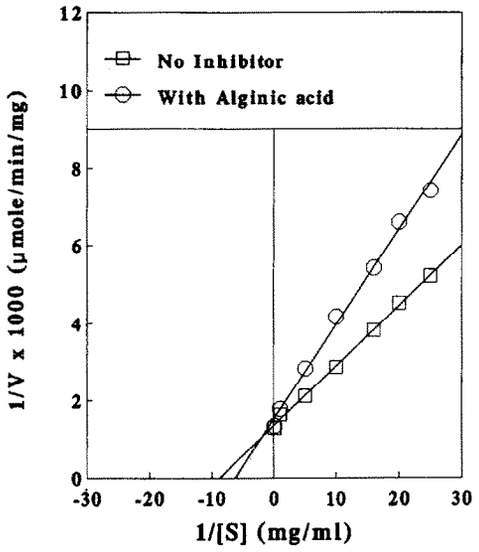

Fig. 10. Lineweaver–Burk plot of papaya pectinesterase activity with alginic acid inhibitor.

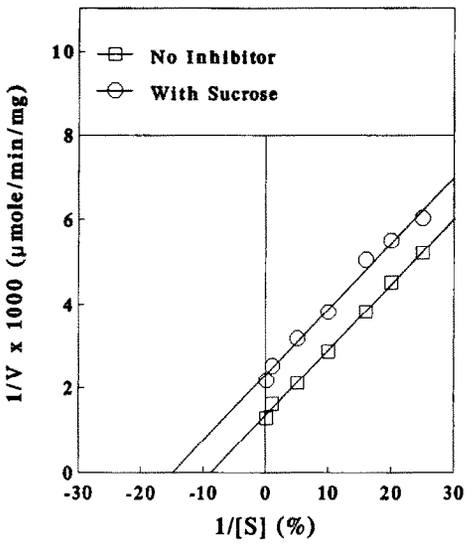

Fig. 11. Lineweaver–Burk plot of papaya pectinesterase activity with sucrose inhibitor.

polygalacturonic acid are quite different. Papaya pectinesterase from the variety exotica is much more strongly inhibited by polygalacturonic acid than pectinesterase from papaya variety solo. This difference in inhibition is supported by the results obtained by Versteeg *et al.* (1978), who obtained different K_i values (0·0016 and 0·416) for the two pectinesterases purified from the navel orange.

The presence of alginic acid in the reaction mixture also shows an inhibitory effect on the pectinesterase activity. Figure 10 shows the enzyme activity measured at different substrate concentrations in the absence and presence of 0·1 mg/ml of alginic acid. Alginic acid shows a mixed type of inhibition termed competitive–non-competitive inhibition, because the pattern observed lies between those for competitive and non-competitive inhibition. The K_i value was calculated by the formula described by Palmer (1981) for such a type of inhibition, and a value of 0·17 mg/ml was obtained for the acid.

The inhibition of papaya pectinesterase (Chang *et al.*, 1965), apple pectinesterase (Lee & Wiley, 1970), banana pectinesterase (Brady, 1976) and *Ficus awkeotsang* makino achenes pectinesterase (Lin *et al.*, 1989) activity by sucrose has been observed, but no attempt was made to determine the K_i value for this inhibitor. Chang *et al.* (1965) described sucrose as a non-competitive inhibitor. In this study an experiment was designed to determine the effect of sucrose on the activity of papaya (var. exotica) pectinesterase. Figure 11 shows the enzyme activity measured at different substrate concentrations in the presence and absence of 20% sucrose in the reaction mixture. Results obtained show that this enzyme is also inhibited by the addition of sucrose. In addition, sucrose was found to act as an uncompetitive inhibitor of papaya (var. exotica) pectinesterase and the K_i value was calculated to be 29%. The actual mechanism involved in which sucrose acts as a pectinesterase inhibitor is not yet known. However, an environment of lower water activity due to the presence of sucrose may be the most important factor involved, since pectinesterase requires water for its activity (Lee & Wiley, 1970). On the other hand, Lin *et al.* (1989) did not agree with this explanation, and their studies on *Ficus awkeotsang* pectinesterase indicated that inhibition by sucrose was not a function of reduced water potential since glycine at the same molar concentration did not show the inhibition. Nor was it clearly related to the total addition of hydroxyl groups by the solute. The same type of explanation was also given by Brady (1976), who studied the effect of organic solutes on banana pectinesterase activity. According to Lin *et al.* (1989), the interaction of sucrose with pectin may alter the hydrophobic packing and change the accessibility of the structure to pectinesterase.

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