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Influence of environmental conditions on thermal stability of recombinant *Aspergillus aculeatus* pectinmethylesterase

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ABSTRACT

The thermal stability of purified recombinant *Aspergillus aculeatus* pectinmethylesterase (PME) in different media was studied. The influence of pH, ionic strength and additives (salts and polyols) was evaluated. At pH 5.0 and a high ionic strength (0.50 M), the enzyme showed a high thermostability (inactivation at temperatures $\geqslant$60 °C). Interestingly, an enhancement of its heat stability was observed at pH 7.0 and temperatures above 55 °C, this behaviour was reflected in an atypical evolution of structural changes in the overall conformation of the enzyme, according to FTIR spectroscopy results. Recombinant *A. aculeatus* PME thermal inactivation at pH 7.0 could be described by a fractional-conversion model. Addition of NaCl increased the thermal stability at pHs 5.0 and 7.0, while addition of $CaCl_2$ had no influence. With regard to sugars (sucrose, trehalose, glucose and maltose) and polyols (sorbitol, lactitol and glycerol) addition, at the same concentration and pH, the polyols showed a higher protective effect than sugars. Also, the thermostability of recombinant *A. aculeatus* PME increased with the additive concentration, although the source of OH groups was the main parameter involved.

1. Introduction

Nowadays, there is a growing interest in enzymes as a useful tool in food processing. Enzymes are often related to quality attributes (such as texture, appearance, flavour and storage stability), which contribute to food acceptability, and are becoming increasingly available for processors to accelerate or enhance the formation of desirable characteristics or to delay the development of undesirable properties (Baker & Wicker, 1996). Pectinmethylesterase (PME, EC 3.1.1.11) is present both in plants and in microorganisms (fungi and bacteria). This enzyme is involved in the modification of pectic substances, which are glycosidic macromolecules of high molecular weight that are the major components of the middle lamella in higher plants (Markovič & Janeček, 2004). PME catalyses the de-esterification of pectin leading to the formation of pectin with a lower degree of esterification and methanol. In general, plant PMEs de-esterify pectins blockwise (single-chain mechanism) whereas acidic microbial PMEs, like *Aspergillus* species, attack the methyl groups on the pectin more randomly (multiple-chain mechanism). De-esterified pectins can create a pectate gel in the presence of calcium ions, rigidifying the cell wall, or can be depolymerised by the action of polygalacturonase (PG, EC 3.2.1.15), contributing to cell wall loosening (D'Avino, Camardella, Christensen, Giovane, & Servillo, 2003; Micheli, 2001).

Most of the commercially available pectic enzymes used in food processing are derived from fungal sources, in particular from *Aspergillus* species (Dalbøge, 1997; Kashyap, Vohra, Chopra, & Tewari, 2001). Microbial enzymes offer the advantage that they can be produced in virtually unlimited quantities and they are potentially inexpensive (Klibanov, 1983). Today, addition of exogenous fungal PME finds a wide application in the fruit processing industry for extraction and clarification of fruit juice, maceration and solubilisation of fruit tissues, and enhancement of fruit texture and firmness (Alkorta, Garbisu, Llama, & Serra, 1998; Degraeve, Saurel, & Coutel, 2003; Jayani, Saxena, & Gupta, 2005; Rombouts & Pilnik, 1986; Whitaker, 1984). Because heat treatment is widespread in the food industry, the knowledge of the thermal stability of enzymes is essential, in order to optimise processing conditions. In this sense, it is necessary to take into account that the stability of an enzyme depends on the environmental conditions (Guiavarc'h, Sila, Duvetter, Van Loey, & Hendrickx, 2003; Gummadi & Panda, 2003; Rexová-Benková & Markovič, 1976; Van den Broeck, Ludikhuyze, Van Loey, Weemaes, & Hendrickx, 1999). Among other factors, medium pH may cause changes in the protein conformation and, hence, affect the thermal inactivation of enzymes.

There have been some studies on the stability of recombinant *Aspergillus aculeatus* PME during processing (Duvetter et al.,

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2005; Dirix, Duvetter, Van Loey, Hendrickx, & Heremans, 2005), but there is a lack of information on the influence of different environmental conditions (pH, ionic strength and presence of salts, sugars and polyols) on the thermal stability of recombinant *A. aculeatus* PME.

2. Materials and methods

2.1. Enzyme source

Recombinant *A. aculeatus* PME was obtained from a commercial preparation (Novoshape, Novozymes, Bagsvaerd, Denmark). Further purification was carried out by gel filtration using an Akta prime system (GE Healthcare, Uppsala, Sweden) with a Hi-Load™ 16/60 Superdex™ 75 prep grade column (GE Healthcare). Fractions containing PME activity were recovered by eluting 5 ml of the commercial preparation (diluted 1/10 in elution buffer) with 50 mM sodium–acetate buffer at pH 4.5 (0.2 M NaCl), at a flow rate of 0.3 ml/min. Analysing those fractions on SDS–PAGE, only one band was observed, corresponding to a molar mass of 41 kDa. This result is in agreement with that reported by Duvetter et al. (2005) for recombinant *A. aculeatus* PME.

2.2. PME activity assay

PME activity was measured by continuous titration (automatic pH-stat 718, Metrohm, Herisau, Switzerland) of the acid production per time unit at pH 4.5 and 22 °C, using 0.01 M NaOH to maintain the pH. The reaction mixture consisted of 250 µl PME and 30 ml of a 3.5 mg/ml apple pectin solution (70–75% esterification, Fluka, Buchs, Switzerland) containing 0.117 M NaCl. The PME activity is proportional to the rate of base consumption, corrected for incomplete dissociation of the demethoxylated carboxyl groups, by multiplying the activity by a factor $[1 + 10^{(pKa-pH)}]$, with pKa equal to 3.5, the pKa of galacturonic acid (Christgau et al., 1996). The activity unit (U) of PME is defined as the amount of enzyme capable of hydrolysing 1 µmol of methoxyl esters per minute, under the above mentioned assay conditions.

2.3. Thermal treatments

Isothermal experiments were carried out in a temperature-controlled water bath. Samples were heated in glass capillaries (1.15 mm × 150 mm, Hirschmann Laborgerate, Eberstadt, Germany), in order to ensure isothermal conditions. After preset time intervals, the capillaries were withdrawn and immediately cooled in an ice–water bath, to stop the effect of heat. The residual PME activity was measured within 60 min of storage in ice–water. No reactivation of the enzyme was observed during this period.

2.4. Effect of environmental conditions

The effect of pH on the thermal stability of recombinant *A. aculeatus* PME was studied in different buffer solutions at concentrations of 0.10 M and 0.50 M. The buffers used were as follows: citric acid buffer at pH values of 3.0, 5.0 and 7.0, sodium–acetate buffer at pH 5.0, sodium–phosphate buffer at pH 7.0 and barbiturate buffer

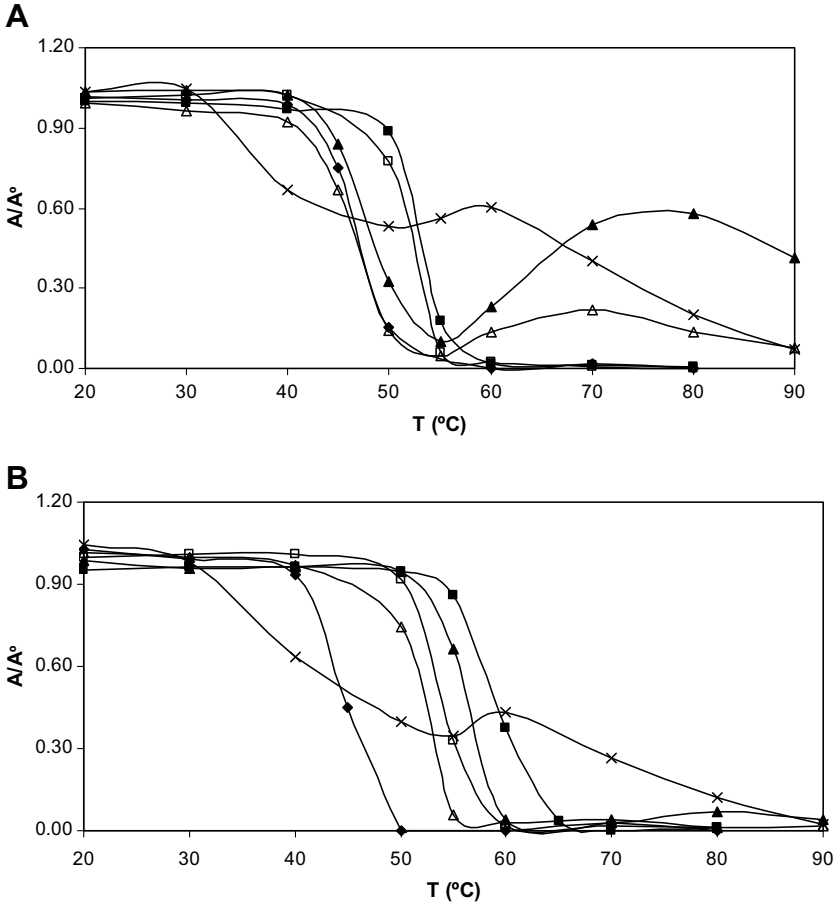

Fig. 1. Residual activity of recombinant *A. aculeatus* PME as a function of pH and molar concentration of buffer salts, 0.10 M (A) and 0.50 M (B), after 10 min of treatment at various temperatures. (♦) citric acid buffer pH 3.0, (■) citric acid buffer pH 5.0, (□) sodium–acetate buffer pH 5.0, (▲) citric acid buffer pH 7.0, (Δ) sodium–phosphate buffer pH 7.0, (×) barbiturate buffer pH 9.0.

at pH 9.0. Also, the influence of the presence of additives was investigated. Sugars such as sucrose (450 and 750 mg/ml), trehalose (450 mg/ml), glucose (450 mg/ml) and maltose (450 mg/ml), polyols such as sorbitol (200 and 450 mg/ml), lactitol (200 mg/ml) and glycerol (45% v/v), and salts such as NaCl (2.4 and 3.4 M) and $CaCl_2$ (0.020, 0.80 and 2.0 M) were evaluated at different pH values.

2.5. Fluorescence spectroscopy

For fluorescence studies, samples were diluted in 0.10 M citric acid buffer at pH 7.0 (final specific activity of the enzyme: 16 U/mg protein), thermally treated at 25, 48, 55 and 75 °C for 10 min and immediately cooled in an ice–water bath. Then, treated samples were used for intrinsic and extrinsic fluorescence measurements. Fluorescence determinations were performed in a Cary Eclipse spectrofluorometer (Varian, Sint-Katelijne-Waver, Belgium).

The ANS (anilino-naphthalenesulfonic acid) probe was used according to the method of Alizadeh-Pasdar and Li-Chan (2000). Briefly, 15 μl of 8 mM ANS (hemimagnesium salt, Sigma, St. Louis, MO) was added to 3 ml of diluted sample solution. The ANS-sample mixture was excited at 350 nm and the fluorescence intensity (FI) was measured at 530 nm. The FI of the sample without ANS was also measured and subtracted to obtain the spectra for the ANS-bound enzyme fluorescence.

For intrinsic tryptophan fluorescence, diluted samples were excited at 295 nm to avoid the excitation of tyrosine and the emission was measured at 340 nm (Ruan & Balny, 2002).

2.6. FTIR (Fourier transform IR) spectroscopy

For FTIR experiments, purified PME was diluted in 0.10 M citrate buffer at pH 7.0, to get a final concentration of 0.2 mg protein/ml. Enzyme samples were thermally treated for 10 min at different temperatures (25, 48, 55, 65, 80 and 90 °C) and immediately cooled in an ice–water bath to stop the effect of heat. The residual activity of PME was measured within 60 min of storage in ice–water. After treatment, samples were dialysed exhaustively against deionised water for 72 h and freeze-dried. Then, they were dissolved in D_2O to a concentration of 50.0 mg/ml and left overnight to ensure that all accessible protons were exchanged for deuterons.

The IR spectra were taken using a Bruker IFS66 (Karlsruhe, Germany) FTIR spectrometer equipped with a liquid–nitrogen–cooled mercury–cadmium–telluride detector. Interferograms (256) were taken at a resolution of 2 cm^{-1} and combined to obtain a good signal-to-noise ratio. Dry air was constantly purged through the spectrometer. Fourier deconvolution was performed using Bruker software. The assumed line shape was Lorentzian. A half bandwidth of 21 cm^{-1} and an enhancement factor of 1.7 were used.

2.7. Data analysis

Inactivation of enzymes can often be described by a first-order kinetic model (Ludikhuyze, Van Loey, Indrawati, Smout, & Hendrickx, 2001),

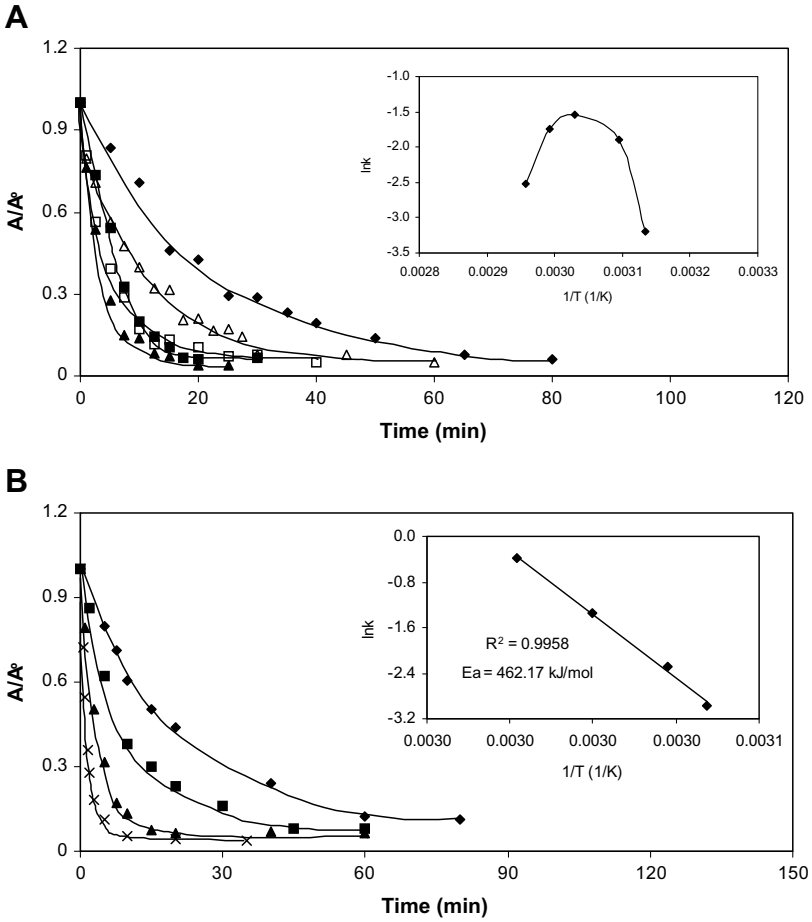

Fig. 2. Residual activity of recombinant *A. aculeatus* PME in 0.10 M (A) and 0.50 M (B) citric acid buffer at pH 7.0 modelled, using a fractional-conversion first-order model. Insert: temperature dependence of the inactivation rate constant. (A) (♦) 46 °C, (■) 50 °C, (▲) 57 °C, (□) 61 °C, (Δ) 65 °C; (B) (♦) 55 °C, (■) 56 °C, (▲) 58 °C, (×) 60 °C.

Table 1
Kinetic parameter estimates of the fractional-conversion thermal inactivation of recombinant *A. aculeatus* PME in 0.10 M and 0.50 M citric acid buffer at pH 7.0

	0.10 M citric acid buffer		0.50 M citric acid buffer	
T (°C)	k (min^{-1})	A_∞ (%)	k (min^{-1})	A_∞ (%)
46	0.05 ± 0.00[a]	3.1	nd	nd
50	0.15 ± 0.02	3.1	nd	nd
55	nd	nd	0.05 ± 0.00	9.5
56	nd	nd	0.10 ± 0.01	9.0
57	0.27 ± 0.01	4.4	nd	nd
58	nd	nd	0.26 ± 0.01	6.0
60	nd	nd	0.69 ± 0.02	5.2
61	0.22 ± 0.01	7.6	nd	nd
65	0.11 ± 0.01	8.8	nd	nd

nd: not determined.
[a] Standard error of regression.

$$A = A_0 \exp(-kt) \quad (1)$$

where A is the enzyme activity at time t, A_0 is the initial enzyme activity, k is the inactivation rate constant and t is the treatment time.

The fractional-conversion model (a special case of the first-order model) takes into account a constant nonzero activity after prolonged processing time (=A_∞) and can be expressed mathematically as

$$A = A_\infty + (A_0 - A_\infty)\exp(-kt) \quad (2)$$

This relation is valid under processing conditions, where only the labile enzyme fraction inactivates, whereas the activity of the stable fraction does not change with time. By plotting A (residual activity after different time intervals) versus time of treatment, the inactivation rate constant (k-value) and the remaining activity after prolonged treatment (A_∞-value) can be estimated using non-linear regression analysis.

The temperature dependence of the inactivation rate constant (k) can be expressed by the activation energy (E_a) and estimated using the Arrhenius equation:

$$k = k_{ref} exp\left\{\frac{E_a}{R_g}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right\} \quad (3)$$

where k_{ref} is the inactivation rate constant at reference temperature (min^{-1}), E_a the activation energy (kJ mol^{-1}), R_g the universal gas

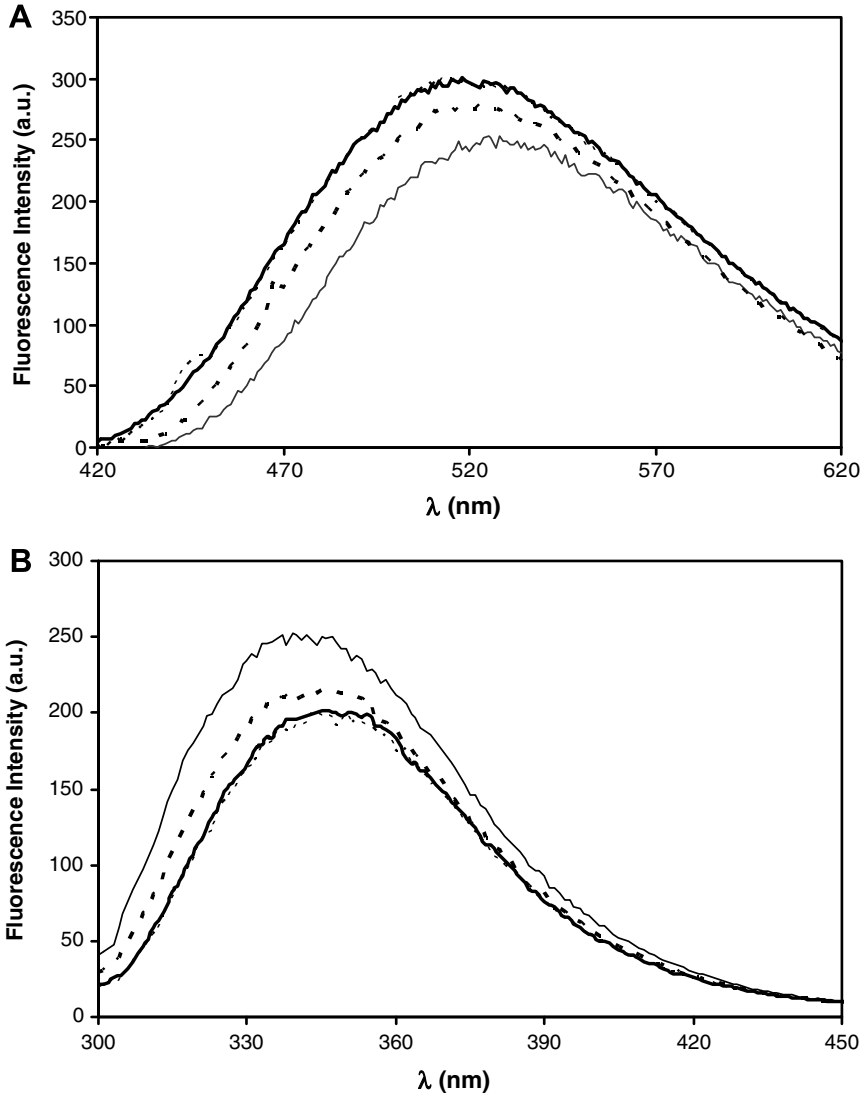

Fig. 3. (A) ANS fluorescence emission spectra, excitation at 350 nm, and (B) tryptophan fluorescence emission spectra, excitation at 295 nm, in the presence of recombinant *A. aculeatus* PME treated at different temperatures in 0.10 M citric acid buffer at pH 7.0. (——) 25 °C, (— — —) 48 °C, (- - -) 55 °C, (——) 75 °C.

constant (8.314 J K^{-1} mol^{-1}) and T_{ref} the reference temperature in (K).

Eq. (3) can be linearised which allows the activation energy to be estimated through linear regression, when the natural logarithm of the rate constant is plotted versus the reciprocal of temperature.

3. Results and discussion

3.1. Effect of pH and ionic strength

3.1.1. Inactivation studies

The thermal stability of recombinant *A. aculeatus* PME was studied in several buffers at two molar concentrations (0.10 M and 0.50 M) over a pH range between 3.0 and 9.0. As shown in Fig. 1A, the thermal inactivation of PME was clearly dependent on the pH of the medium. The maximum thermostability of the enzyme (at 50 °C) was observed at pH 5.0, which is close to the pH optimum (4.5) for its catalytic activity (Christgau et al., 1996). When the pH increases or decreases from 5.0 a decrease in thermal stability is observed. Interestingly, at pH 7.0 and temperatures above 55 °C, the enzyme showed an apparent increase in heat stability, which was more pronounced when citric acid buffer was used. However, at higher ionic strength (Fig. 1B) the above mentioned phenomenon was less pronounced. A similar behaviour was found at pH 9.0 but less obvious. On the other hand, when the molar concentration increased from 0.10 to 0.50, an increase in thermal stability at pH 5.0 and pH 7.0 was observed, whereas at both sides of this pH range the stability decreased slightly.

The thermal inactivation of recombinant *A. aculeatus* PME at pH 7.0 could be accurately described by a fractional-conversion model (Figs. 2A and B). However, it has been reported that a first-order model could be used to estimate the kinetic inactivation parameters of this enzyme at pH 4.5 (Duvetter et al., 2005), indicating the significance of pH. Inactivation rate constants were estimated, using nonlinear regression analysis of Eq. (2), and are reported in Table 1. The k values at the highest ionic strength increased with temperature while those at the lowest ionic strength increased only up to 57 °C and then decreased. Hence, at pH 7.0 and the lowest ionic strength the law of Arrhenius does not apply for the inactivation of this PME, which is very unusual in enzyme inactivation. At similar temperatures, the k values at the lowest ionic strength, were higher compared to those at the highest ionic strength indicating that recombinant *A. aculeatus* PME is less thermostable at low ionic strength. These results confirm those obtained by the thermostability screening (Figs. 1A and B). To shed some light on the unusual inactivation behaviour of recombinant *A. aculeatus* PME, some investigations of the protein structure after thermal treatments were performed.

3.1.2. Fluorescence spectroscopy

The catalytically active enzyme structure is maintained by a balance of different non-covalent forces, mainly hydrogen bonds, hydrophobic interactions, electrostatic interactions and van der

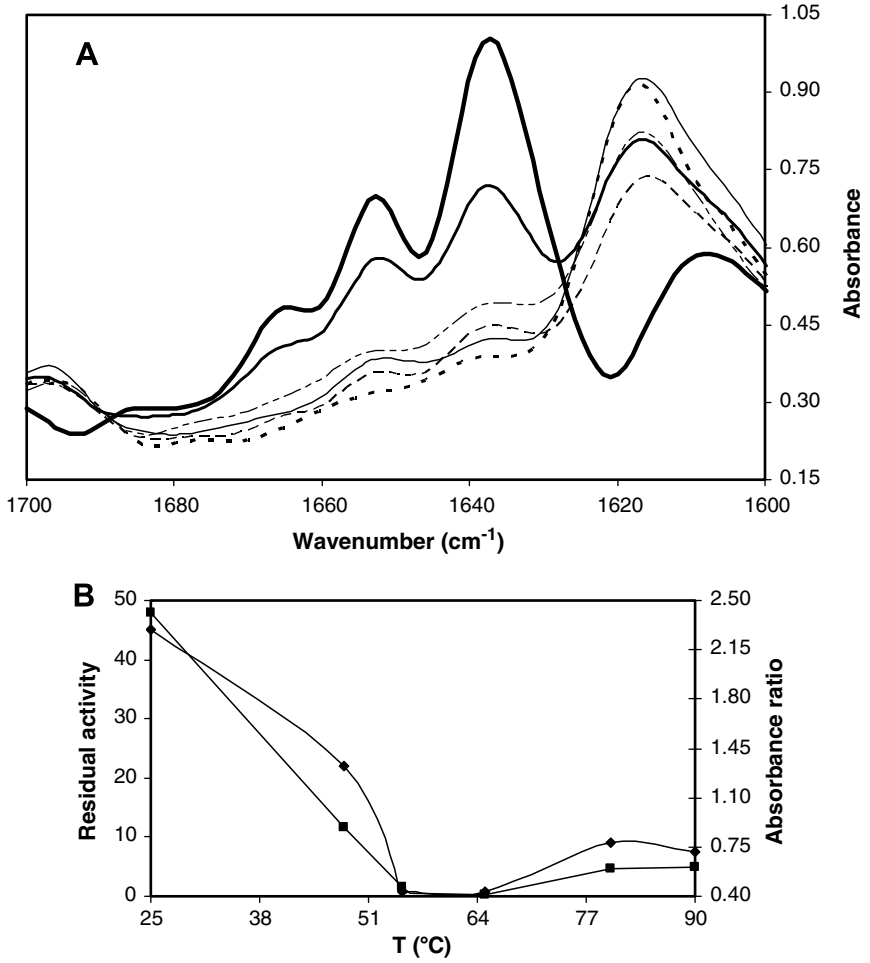

Fig. 4. (A) Deconvoluted amide I' band of recombinant *A. aculeatus* PME treated at different temperatures in 0.10 M citric acid buffer at pH 7.0. (▬) 25 °C, (▬) 48 °C, (▬) 55 °C, (– –) 65 °C, (— - -) 80 °C, (— —) 90 °C. (B) Residual activity and absorbance ratio of the band at 1637 cm^{-1} versus the band at 1617 cm^{-1} of recombinant *A. aculeatus* PME treated at different temperatures in 0.10 M citric acid buffer at pH 7.0. residual activity (♦), absorbance ratio (■).

Waals forces (Klibanov, 1983). The contribution of hydrophobic interactions to the thermal stabilisation of proteins has been considered to be of great importance (Longo & Combes, 1997). In the present work, the influence of these forces was evaluated using the ANS probe. The fluorescence emission of ANS increases in a hydrophobic environment while ANS has a lower yield in hydrophilic surroundings. Fig. 3A shows the fluorescence emission spectra of ANS in the presence of PME treated at pH 7.0 and different temperatures. The fluorescent probe showed an increase in FI with the treatment temperature, until a maximum was reached at 55 °C, indicating enzyme unfolding, as nonpolar residues become exposed to the solvent and, hence, an increase of the surface hydrophobicity occurs. Also, the emission maximum of ANS underwent a blue shift from 528 to 515 nm, due to the increase of environment hydrophobicity. However, it is necessary to take into account that ANS is anionic and electrostatic interactions with the enzyme cannot be excluded. Tryptophan fluorescence has been at medium polarity allows the detection and monitoring of protein conformational changes (Ruan et al., 2002). As shown in Fig. 3B, between 25 and 55 °C FI of the enzyme decreased and the emission maximum shifted from 339 to 346 nm (red shift) because of greater exposure of tryptophan residues to the aqueous solvent when the enzyme gradually unfolds. However, a further increase in temperature between 55 and 75 °C did not show any fluorescence change for the ANS probe or tryptophan fluorescence. Therefore, the enhancement of PME thermal stability at temperatures above 55 °C does not seem to be related to internal hydrophobic interactions. However, there is no information on intermolecular hydrophobic interactions.

If no other forces (apart from electrostatic forces) are involved, an enzyme is most stable at its isoelectric pH, at which attractive and repulsive electrostatic forces are balanced. These interactions are pH-dependent and, hence, pH can alter the state of ionisation of amino acids in an enzyme, affecting its functioning. Thus, some side chains of amino acids can act as weak acids or bases and play a main role in the active centre (Jyothi, Singh, & Rao, 2005). Ionic interactions could be involved in the increase of stability observed at pH 7.0 and temperatures above 55 °C.

3.1.3. FTIR spectroscopy

Usually, during enzyme inactivation, a global conformational change takes place. Changes of the secondary structure of the enzyme can be monitored using FTIR spectroscopy. The deconvoluted FTIR spectra (amide I' region: 1600–1700 cm^{-1}) of recombinant *A. aculeatus* PME, recorded after treatment at different temperatures at pH 7.0, are shown in Fig. 4A. The main secondary structure elements of the native enzyme (at 25 °C) were β-sheet (1637 cm^{-1}), α-helix (1652 cm^{-1}) and turns and bends (1665 cm^{-1}), with the β-sheet being the predominant structural unit. These structure elements are in agreement with those reported by Dirix et al. (2005), and are indicative of the β-helix structure. When the temperature of treatment increased from 25 °C to 55 °C, the intensity of the bands corresponding to β-

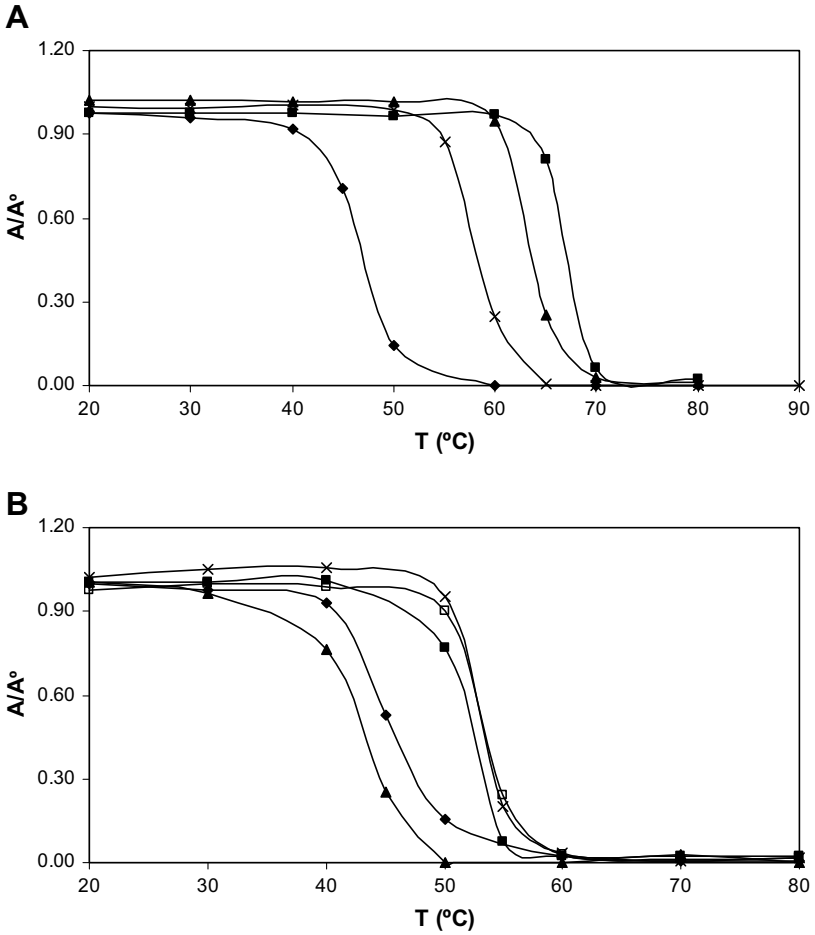

Fig. 5. Residual activity of recombinant *A. aculeatus* PME as a function of pH and salt concentration after 10 min of treatment at various temperatures. (A) 0.10 M citric acid buffer at pH 3.0 (♦), 5.0 (■, ▲) or 7.0 (×) containing 2.40 M (♦, ▲, ×) or 3.40 M (■) NaCl. (B) 0.10 M citric acid buffer at pH 3.0 (♦, ▲) or 0.10 M sodium–acetate buffer at pH 5.0 (■, □, ×) containing 0.020 M (♦, ■), 0.80 M (▲, □) or 2.0 M (×) $CaCl_2$.

sheet, α-helix and turns and bends decreased, indicating protein unfolding. At the same time, a band indicative of intermolecular aggregation (1617 cm^{-1}) appeared and increased. On the other hand, at temperatures above 65 °C, a higher intensity of bands corresponding to β-sheet and α-helix was noticed, whereas the intensity of the band indicative of aggregation was lower. Fig. 4B shows the residual activity of PME and the absorbance ratio of the band at 1637 cm^{-1} (β-sheet, the most pronounced band indicative of the native structure) *versus* the band at 1617 cm^{-1} (aggregation, the most pronounced band indicative of denatured structure) after thermal treatments. Both the residual activity and the absorbance ratio of the bands, exhibited a similar behaviour that was confirmed by a strong correlation (r^2 = 0.9354). Hence, the remaining level of enzyme activity after a thermal treatment was proportional to the remaining level of native protein structure at all temperatures studied. The atypical

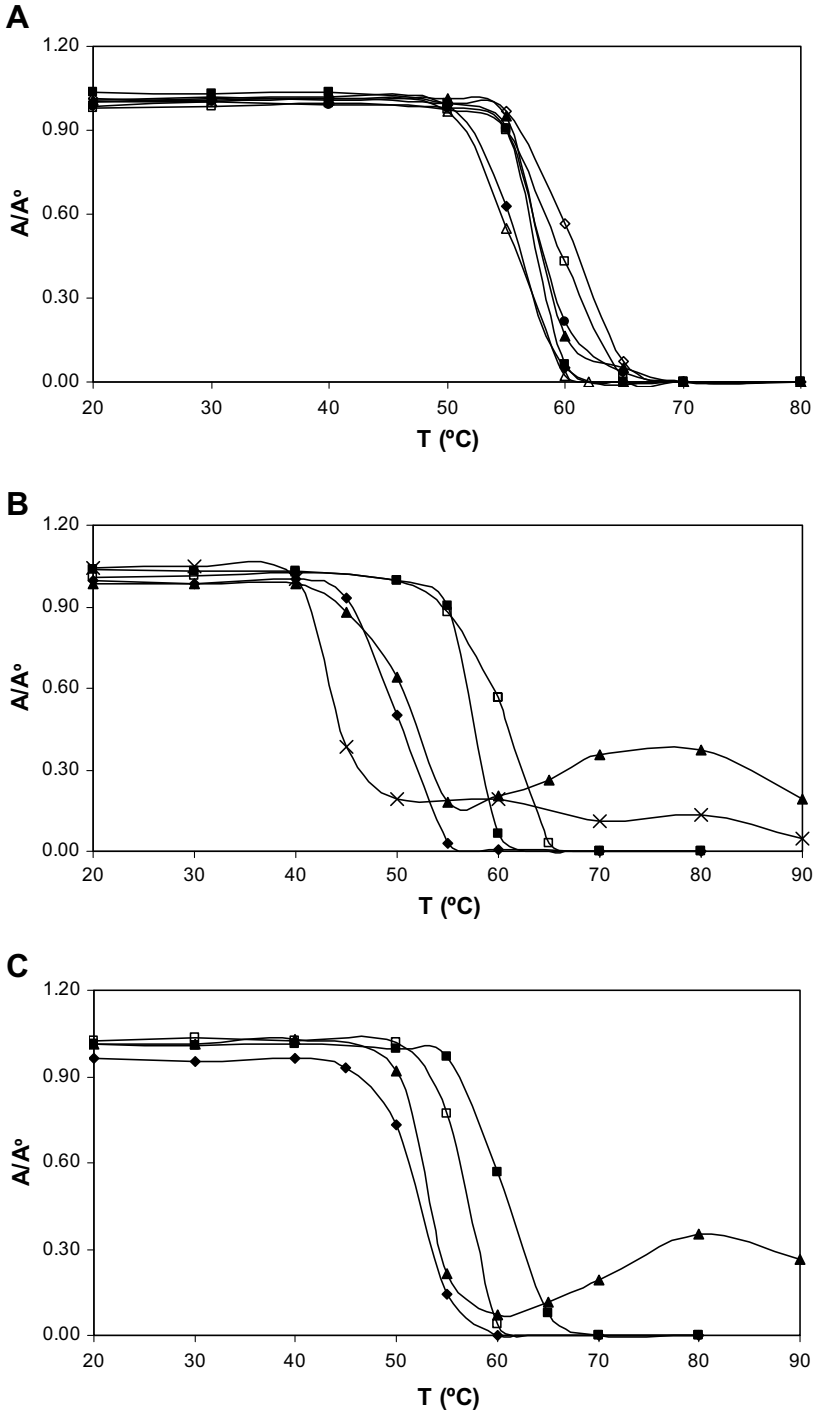

Fig. 6. Residual activity of recombinant *A. aculeatus* PME as a function of pH and different additives after 10 min of treatment at various temperatures. (A) As a function of additive at pH 5.0 (0.10 M citric acid buffer): (■) 450 mg/ml sucrose, (▲) 450 mg/ml trehalose, (●) 450 mg/ml glucose, (♦) 450 mg/ml maltose, (◇) 450 mg/ml sorbitol, (Δ) 200 mg/ml lactitol, (□) 45% v/v glycerol; (B) as a function of pH (0.10 M citric acid buffer at pH 3.0, 5.0 and 7.0, or 0.10 M borate buffer pH 9.0) and concentration of sucrose: (♦) pH 3.0 and 450 mg/ml, (■) pH 5.0 and 450 mg/ml, (□) pH 5.0 and 750 mg/ml, (▲) pH 7.0 and 450 mg/ml, (×) pH 9.0 and 450 mg/ml; (C) As a function of pH (0.10 M citric acid buffer at pH 3.0, 5.0 and 7.0) and concentration of sorbitol: (♦) pH 3.0 and 450 mg/ml, (■) pH 5.0 and 450 mg/ml, (□) pH 5.0 and 200 mg/ml, (▲) pH 7.0 and 450 mg/ml.

Table 2
Number of hydroxyl groups provided by sugars and polyols at different concentrations

nOH/ml × 10^{22}	Maltose (mg/ml)	Trehalose (mg/ml)	Sucrose (mg/ml)	Glusose (mg/ml)	Lactitol (mg/ml)	Sorbitol (mg/ml)	Glycerol (% v/v)
0.299					200		
0.397						200	
0.602	450						
0.633		450	450				
0.684				450			
0.893						450	
1.056			750				
1.115							45

inactivation behaviour was reflected in an atypical evolution of structural changes in the overall conformation of the enzyme.

3.2. Effect of salts

Salts can affect the thermal stability of an enzyme in several ways, depending on the environmental conditions, the ion and the enzyme involved. In our work, high concentrations of NaCl significantly stabilised PME at pHs 5.0 and 7.0, while no effect was observed at pH 3.0 (Figs. 1A and 5A). This protection of the native structure has been related to decreasing enzyme solubility, by enhancing the driving force for the transfer of nonpolar groups away from contact with the solvent (Phillips, Whitehead, & Kinsella, 1994). Regarding the thermal stability in the presence of NaCl at neutral pH, there is complete inactivation at 65 °C and curiously no increase of stability at temperatures above 65 °C. This observation indicates the importance of ionic interactions in protein stability. On the other hand, $CaCl_2$ had no influence on the thermal stability of PME at pH 3.0 and 5.0, except for a small decrease observed at the highest concentration studied and pH 3.0 (Figs. 1B and 5B). These results are in agreement with those found by Duvetter et al. (2005) for recombinant *A. aculeatus* PME at pH 3.8, showing that the presence of Ca^{2+} ions has no influence on the inactivation rate of the enzyme.

3.3. Effect of sugars and polyols

Additives, such as sugars and polyols, can be used in order to enhance the stability of enzymes in aqueous solutions. Sugars and polyols have been reported to protect the native conformation of enzymes against thermal denaturation by augmenting the structure of water surrounding the molecule (Phillips et al., 1994). To study the influence of these additives on the thermal stability of recombinant *A. aculeatus* PME, several sugars (sucrose, trehalose, glucose and maltose) and polyols (sorbitol, lactitol and glycerol) were evaluated at different concentrations and pH. As shown in Figs. 6A and B, the stability of PME in the presence of sugars slightly increased, compared to that without additives (Fig. 1A). The most positive effect was found at pH 5.0, the stabilising influence of sugars decreased when the pH moved away from that value. There was no effect of sugar type at the same concentration and pH. For all sugars tested the enzyme stability increased with increased concentration. Polyols showed a similar behaviour but their protective effect was higher in relation to sugars (Figs. 1B, 6A and C). However, the addition of sucrose or sorbitol did not influence the atypical heat inactivation at pH 7.0.

The number of hydroxyl groups (nOH) provided by additives has been related to the thermal stability of enzymes (Haque, Singh, Moosavi-Movahedi, & Ahmad, 2005; Obon, Manjon, & Iborra, 1996). Guiavarc'h et al. (2003) reported that the thermostability of tomato PME in presence of different additives was determined by the nOH instead of the source of these groups. However, Samborska, Guiavarc'h, Van Loey, and Hendrickx (2006) observed that the heat stability of *Aspergillus oryzae* α-amylase was not dependent on the amount of hydroxyl groups provided by additives. They reported that sucrose exhibited the largest protective effect. In the present work, polyols showed higher stabilising power than sugars at the same concentration and pH. Taking into account the nOH provided by sugars and polyols (Table 2), it seems that the nOH per molecule and the total nOH are not related to the thermal stability of recombinant *A. aculeatus* PME. Although the concentration of additive is important, the main parameter influencing its thermostability is the source of OH groups, the highest protection being observed when polyols were added. This protection offered by additives is generally attributed to their preferential exclusion from the enzyme surface (their binding would increase the free energy of the system), being related to a solvophobic effect in the case of polyols (Lopez & Burgos, 1995).

4. Conclusion

The present study clearly shows the importance of environmental conditions on the thermal stability of recombinant *A. aculeatus* PME. This knowledge is essential in order to optimise its use as an exogenous enzyme in fruit and vegetable thermal processing. Recombinant *A. aculeatus* PME showed the highest thermostability (at 50 °C) in the presence of NaCl at pH 5.0. Also, increasing the ionic strength, adding sugars and adding polyols had a positive effect on heat stability, whereas the effect of adding $CaCl_2$ was negligible. The effect of pH was found to play a major role. Interestingly, an increase of the heat stability of this enzyme was observed at pH 7.0 and temperatures above 55 °C. This behaviour was reflected in an atypical evolution of structural changes in the overall conformation of the enzyme. Also, ionic interactions could be of importance. Further research is needed to get more insight in the mechanism involved in this change of stability at neutral pH.

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