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Food Chemistry 101 (2007) 1–9
Food Chemistry
www.elsevier.com/locate/foodchem

Hydrolysis of apple pectin by the coordinated activity of pectic enzymes

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Received 23 May 2005; received in revised form 15 December 2005; accepted 15 December 2005

Abstract

The hydrolysis of pectin from apples, cv. Budimka fruit (*Pyrus mahus* L.), by individual and/or combined action of fungal polygalacturonase from *Aspergillus niger* (FPG), fungal pectin esterase from *A. niger* (FPE) and plant tomato pectin esterase (PPE) was studied. The optimum pH values for individual actions of FPG, FPE and PPE on 1% apple pectin were determined to be 4.5, 3.5 and 6.5, and the optimum temperatures were 40, 45, and in the range 45–50 °C, respectively. FPE was found to be more efficient for the hydrolysis of the apple pectin than was PPE from tomato. By measuring the initial velocities on 1% apple pectin it was confirmed that the PG expressed no effect on the PE activity. By using the combination of FPG (162 U/l) and FPE (60 U/l), e.g., in a respective ratio of 2.5, an efficient pectin degradation process, with a viscosity reduction of $\eta/\eta_0 = 1.05$, could be reached in less than 2 h. This process produced about 160 ppm of methanol in the pectin digest. The long term hydrolysis reaction of the apple pectin with FPG (162 U/l) and FPE (27 U/l) achieved a degree of hydrolysis of around 29% after 12 h and consisted mostly of trimers (28.4%).

Keywords: Apple pectin; Kinetics of hydrolysis; Fungal polygalacturonase; Tomato pectin esterase; Fungal pectin esterase; *Aspergillus niger*

1. Introduction

Pectins are very complex and heterogeneous molecules which are regular ingredients of all higher plants. Together with cellulose and hemicellulose, they build the cell walls and contribute to many cell wall functions (Schols & Voragen, 2002). The pectin contains so-called ''smooth regions'' and ''hairy regions''. The smooth regions, also known as homogalacturonan, consist of α(1,4)-linked D-galacturonic acid residues, whereas the hairy regions, or rhamnogalacturonan I are characterized by stretches of alternating α(1,4)-linked D-galacturonic acid and L-rhamnose (Ridley, O'Neill, & Mohnen, 2001; Schols & Voragen, 2002). Various amounts of the galacturonic acid regions are present (methyl esterified), and they could greatly influence the physicochemical properties of the pectin (Schols, Huisman, Bakx, & Voragen, 2003).

Besides their functions in living tissues, pectins are also of commercial interest, as they are used as gelling agents in the manufacture of jams, jellies, marmalades and confectionery and for the stabilization of acidified dairy drinks (May, 2000). In addition, pectin fibres may be used for preparation of various dietary products which could be useful in the prevention of hyperlipidemia as well as large bowel cancer (Kritchevsky & Bondfield, 1997). Some pectin derivatives have been used for preparation of polysaccharide-based vaccines (Szu, Bystricky, Hinojosa-Ahumada, Egan, & Robbins, 1994) and for drug delivery pharmaceuticals (Liu & Krishnan, 1999).

Significant changes related to the characteristics and technological properties of fruit and vegetable products, during maturation, storing and canning, are related to the physicochemical transformations of pectins caused by the action of pectic enzymes. Mostly due to the pectin complexity, the pectic enzymes are comprised of a diverse group of enzymes.

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doi:10.1016/j.foodchem.2005.12.053

They generally, consist of main-chain depolymerases and esterases, active on methyl- and acetylesters of galacturonosyl uronic acid residues in the galacturonan and rhamnogalacturonan structures. The depolymerizing enzymes are comprised of hydrolases as well as lyases (Benen, Voragen, & Visser, 2003a). In recent years, knowledge of the pectic enzymes, both regarding their mode of action (Benen, van Alebeek, Voragen, & Visser, 2003b) and their three dimensional structures (Petersen, Kauppinen, & Larsen, 1997; Van Santen et al., 1999) has radically increased. Significant progress has been achieved as a result of cloning and overexpression of genes encoding pectic enzymes (Benen et al., 2003b). However, data related to the catalytic activity of the specific groups of pectic enzymes from various origins acting on the particular substrates, which are needed for practical purposes, are still of great interest. In addition, our practical experience on the influence of polygalacturonase on the activity of pectin esterase and vice versa in the real systems is still insufficient.

Both naturally present (endogenous) and introduced enzymes (exogenous) catalyze the decomposition of pectins. As processing aids, pectic enzymes have predominantly found application in the fruit juice industry (Benen, van Alebeek, Voragen, & Visser, 2003c; Pilnik & Voragen, 1993). Commercial enzymatic preparations used for pectin degradation, usually contain fungal polygalacturonase (FPG) as a basic depolymerization enzyme, which catalyzes the hydrolysis of partially-esterified pectin compounds, meaning that pectin esterase is necessary for the successful depolymerization. For as complete degradation of pectin substrate as possible, with minimal release of methanol (MeOH), it is necessary to optimize the PG and PE ratio. It is important to note that the level of MeOH released during pectin degradation is strictly regulated by appropriate technological standards (Pilnik & Voragen, 1993).

The main goal of the present study was to examine and optimize the process of enzymatic degradation of apple pectin derived from our autochthons Budimka apple variety (*Pyrus malus* L.). For this purpose, the concentration and the ratio of pectolytic enzymes from *Aspergillus niger* (fungal polygalacturonase – FPG and fungal pectin esterase – FPE) and from tomato (tomato plant pectin esterase – PPE) and the kinetics of the enzymatic reaction were investigated. The process conditions, such as time, temperature, pH and the levels of the released MeOH were optimized in order to attain an appropriate chemical composition and technological properties of the products during fruit processing.

2. Materials and methods

2.1. Pectin extraction and purification

The apple variety Budimka (*P. malus* L.), from the Arilje locality (Western Serbia), collected during 2002, was used in the investigation. Polygalactouronic acids, extracted from the Budimka apple were isolated and purified by the modified method described by Mukhiddinov, Khalikov, Abdusamiev, and Avloev (2000). Apples were first washed and minced in an electric grinder. The crushed pulp was then pressed and the mash was dried, initially at room temperature and then at 50 °C, to a constant weight. Obtained dried apple pulp was then crushed and pulverized, by mixing, and used as a raw material for further isolation of the PGA rich fraction by treatment with a mixture of 70% alcohol, concentrated HCl, and water in a ratio 14:3.6:2.4, respectively, at room temperature for 24 h. After this, it was washed with 70% alcohol until the reaction for Cl^- ions was negative, and then with 96% alcohol and with acetone, and dried. To eliminate a micro gel (a high molecular mass of the cross-linked polymer produced by the degradation of protopectin) a 0.5% solution of the pectin was centrifuged at 12000*g* for 1.5 h. Finally, the purified pectin obtained was separated on ion-exchange resin DEAE-52 cellulose (Serva) and eluted with bidistilled water. The eluate was concentrated under vacuum and lyophilized. The resulting pectin preparation had a degree of esterification (DE) of 95% (w/v), an average degree of polymerization (DP) of 134, and a polygalactouronic acid content (PGA) of 93% (w/v). The pectin characteristics regarding high DP and DE are rather unusual, and they are typical of Budimka (Arilje locality) apple variety. The apples were in the stage of technological maturity (not completely ripened), which might also contribute to the higher DE and DP values.

2.2. Enzymes and chemicals

Fungal polygalacturonase (FPG) from *A. niger* (Merck, Darmstadt, Germany, declared activity of 1.0 U/mg) and fungal pectin esterase (FPE) from *A. niger* (Rohm and Haas Co., Heidelberg, Germany, declared activity of 1.84 U/mg) were used in this study.

The enzymes were further purified to remove (possibly present) pectin esterase (for FPG) or polygalacturonase (for FPE) by column chromatography with hydroxy apatite as described by Harman and Cordes (1972). The elution was performed with a gradient of 0–1.2 M KCl in 0.01 M phosphate buffer (pH 6.0). In this way, the polygalacturonase was eluted, while the pectin esterase was retained in the column.

Plant tomato pectin esterase (PPE) of high purity was supplied by the Sigma Chemical Co. (Deisenhofen, Germany, declared activity of 35 U/mg).

Standard methods for enzyme activity (PG or PE) presented in the enzyme assays section on apple pectin as a substrate (1% PGA) were used to detect the side activities of the enzymes. No side effects were noticed in purified enzymes.

D(+)-galacturonic acid monohydrate from Sigma Chemical Company (St Louis, USA) was used as the standard. Sugars and sugar alcohols were purchased from Merck (Darmstadt, Germany). All other chemicals used were analytical grade.

2.3. Enzyme assays

The polygalacturonase activity. The polygalacturonase activity (PG) was assayed by measuring the reducing groups released from polygalacturonic acid (1% apple PGA) by a modified arsenic-molybdate photometric method (Somogyi, 1952). The absorption was measured at 520 nm. Calculations were made using a calibration curve. The enzymatic reaction was stopped by the incubation of the reaction mixture at 100 °C for 10 min in a boiling water bath. One unit of PG activity was defined as that amount of enzyme which would yield 1 μmol reducing sugars per minute at 40 °C and pH 4.5.

Viscometric measurements. The progress of the enzymatic reaction was also evaluated by following the viscometric reduction in apple pectin substrates. The flow time of a 10 ml sample was measured at 30 °C in a #100 Ostwald–Finske capillary viscosimeter. The relative, η_r, viscosity was calculated from the following relation:

$$\frac{\eta}{\eta_0} = \left(\frac{t}{t_0}\right)\left(\frac{\rho}{\rho_0}\right) = \eta_r \quad (1)$$

where t is the flow time for the macromolecular solution, and t_0 is the flow time for the solvent in seconds. Because of the low concentration used, (ρ/ρ_0) is usually taken as unity (Moriss, Foster, & Harding, 2002). In certain experiments, the time needed to achieve an estimated degree of the substrate hydrolysis was followed.

Pectin esterase. The pectin esterase activity (PE) was evaluated by titration of the substrate (1% apple PGA) with 0.01 M of NaOH. One Unit of PE activity was taken as the amount of NaOH (μM) consumed per min to keep a constant pH value.

2.4. Enzymatic degradation of apple PGA

Enzymatic degradation of apple PGA (1% w/v) was performed in Erlenmeyer flasks in a thermostated water bath with shaking ($v = 150$ rpm). The reaction mixture, usually containing 1% PGA (w/v), 0.15 M NaCl in acetate buffer (0.02 M) and various amounts of individual or combined enzymes, was incubated at 40 °C (unless otherwise stated). The progress of the reaction was monitored by sampling at the preset time intervals in order to monitor the substrate degradation parameters, as well as the enzyme kinetic parameters, such as enzyme activity and enzyme velocity. Enzymatic reaction of the samples was terminated by heating for 10 min at 100 °C.

2.5. Enzyme activity as a function of temperature and pH

The effect of temperature on the activities of the assayed enzymes was studied by exposing the enzyme solutions to different temperatures (20–60 °C) and various times. Solutions were then cooled and the residual activity measured by the methods described below. The effect of pH on enzyme activities was also studied.

2.6. Analytical methods used to monitor the substrate degradation

The anhydrogalacturonic acid (AGA) content in pectin preparations was determined by the photometric method with carbazole in 80% sulfuric acid and borate ions added. The absorbance of the solutions is compared to that obtained from standard solutions of galacturonic acid subjected to the same procedure (Taylor & Buchanan-Smith, 1992).

Methanol content was determined using chromotropic acid (International Standards ISO 1388/1). The principle is based on the conversion of methanol present in a test portion to formaldehyde by oxidation. The oxidation was carried out with a solution of potassium permanganate in phosphoric acid. Reaction of the formaldehyde formed with chromotropic acid (4,5-dihydroxynaphthalene-2, 7-disulphonic acid). Photometric measurements of the violet coloration obtained were performed at a wavelength of 570 nm.

Degree of methyl esterification of substrate (DM) was calculated on the basis of determined methanol content and anhydrogalacturonic acid (AGA) content and expressed as moles of methanol present per 100 moles of AGA.

Degree of polymerization of substrate (DP) was estimated by determining the ratio of AGA to the reducing group content (AGA/CHO) according to Liu and Luh (1978).

The protein content was determined by Biuret protein spectrophotometric assay. The principle of the method is formation of the coloured complex of proteins from the samples with copper salts in the reagent which could be detected at 280 nm (Layne, 1957).

Thin-layer chromatography (TLC) of oligogalacturonic acids was performed on cellulose TLC plates (Eastman E-13255). Prior to application, the hydrolysate was treated with cation exchange resin, Dowex-50 (H+ form). Each spot contained 35–85 μg (depending on the experiment) of the sample. The plates were developed in an ascending direction at 23 °C with ethyl acetate:acetic acid:water (4:2:3. v/v/v). The spots where visualized by spraying the plates with 10% ammonia solution followed by bromophenol blue (Liu & Luh, 1978). For quantitative assay, an aliquot of 85 μg of hydrolysate was applied for each spot. Quantitative analysis of the oligogalacturonic acid was done by scraping of the acidic spots from the thin-layer plates and analyzing for the content of anhydrogalacturonic acid by the carbazole method described above.

3. Results and discussion

3.1. Effect of pH on enzyme activity

Effect of pH on the activity of pectolytic enzymes usually present in fruit juices is shown in Fig. 1. The effect of

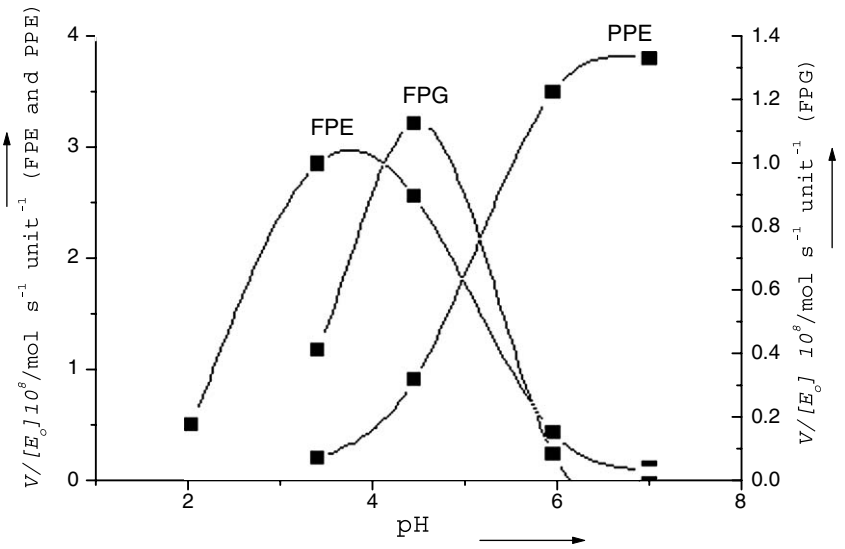

Fig. 1. The effect of pH on the activity of pectolytic enzymes. Reaction conditions: FPG - 615 U/l, 1.0% PGA, 0.15 M NaCl, 0.02 M acetate buffer; FPE – 28 U/l, 1% PGA and 0.15 M NaCl 0.02 M acetate buffer; PPE – 25 U/l, 1% PGA and 0.15 M NaCl, 0.02 M acetate buffer.

pH was studied in acetate buffer. The optimum pH values for FPG, FPE and PPE obtained were close to 4.5, 3.5 and 6.5, respectively (Fig. 1). Recently, several polygalacturonases have been characterized biochemically. The most comprehensive analyses have been carried out for PGs from the *Aspergillus* genus, specifically for the seven-membered endo-PG family from *A. niger* N400 and for the endo-PG family from *A. tubingensis* NW752 (Benen, Kester, & Visser, 1999; Benen et al., 2003b). The optimal pH range for the above seven EPs in McIlvaine buffer (0.25% PGA substrate) was from 3.8 to 5.0. The FPG, in our study, exhibited an optimum pH close to that of the cloned PG I (pH 4.2), PG II (pH 4.2), and PG D (pH 4.2) and also not far from constitutively-expressed PGA and PGB (pH optimum 4.0 and 5.0, respectively) (Benen et al., 2003b). The FPG, studied here, could consisted of a mixture of several PGs produced by *A. niger*, and most probably of those which are constitutively expressed.

The PPE from tomato exhibited significantly higher pH optimum (6.5) than did the FPE (3.5).

3.2. Effect of temperature on enzyme activity

Thermal stability of polygalacturonase and pectin esterase is depicted in Fig. 2. The initial reaction rate was measured immediately upon addition of the enzyme to the reaction mixture. For FPG, the optimum occurs at 40 °C. From 30 to 40 °C the rate exponentially increases. At about

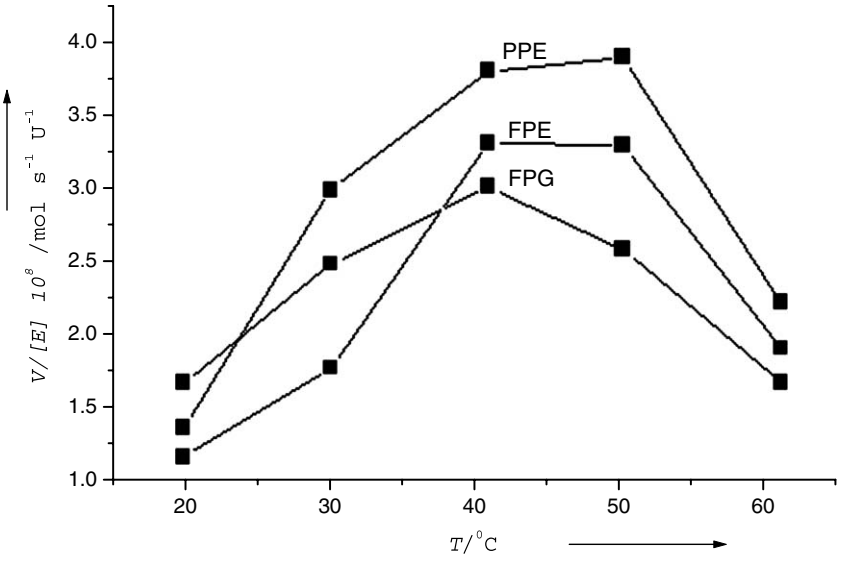

Fig. 2. Effect of temperature on the activity of pectolytic enzymes. Experiments with FPE and PPE in concentrations of 28 U/l, 1% PGA, 0.15 M NaCl, 0.02 M acetate buffer, at optimum pH. FPG activity assays reflected a mean velocity over a 15 min interval. The reaction conditions for FPG were: 615 U/l, 1% PGA, 0.15 M NaCl, 0.02 M acetate buffer at optimum pH, 30 min at different temperatures.

50 °C the activity drops rapidly. Esterases, both fungal and plant, exhibited higher temperature optima than did PG. The optimum temperature for FPE occurs at 45 °C. The optimum temperature interval for PPE is from 45 to 50 °C. Plant esterase showed a slightly higher temperature stability than the fungal one. Generally, all three enzymes tested showed remarkable activity at 40 °C, which was chosen for the temperature which should be applied for their combined action.

3.3. Degradation of apple pectin by FPG

Substrate degradation by FPG from *A. niger* was monitored by simultaneous analyses of the reducing groups and the viscosity of the substrate. The results obtained by analyzing digests of 1% PGA incubated for 30 min with different enzyme concentrations at pH 4.5 are shown in Fig. 3. As shown, the content of reducing groups linearly increased with increasing enzyme concentration, while the viscosity of hydrolysate decreased non-linearly. About 50% of the overall viscosity decrease was observed for the initial 9% hydrolysis of the substrate. Maximal production of the reducing groups (60 μM) was accomplished with 265 U of FPG/l. The relative viscosity value achieved by this amount of FPG was 1.7. Further increase of the amount of enzyme gave limited relative viscosity decrease (data not presented). The results obtained suggested a mechanism of random cleavage of pectin molecules, which is characteristic for endo-PG. This was also supported by the gradual production of oligomers with a decreased degree of polymerization during enzymatic pectin degradation as presented in Table 1. Oligomers with a low degree of polymerization are present at the end of the pectin degradation process. In contrast to endo-PG, the exo-PG cleaves off galacturonic acid monomers or digalacturonic acid from the non-reducing end, resulting in the instant accumulation of small hydrolytic units at the beginning of hydrolysis. The gradual product progression pattern

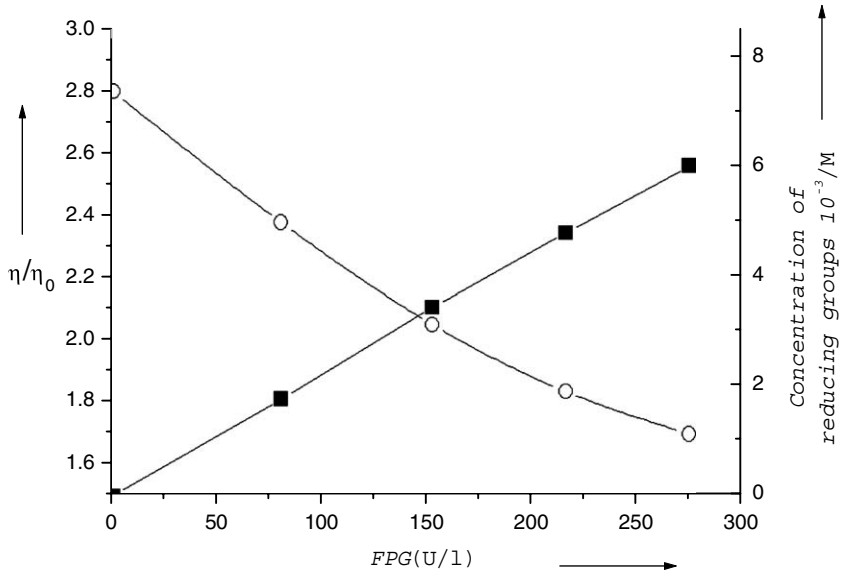

Fig. 3. Hydrolysis of polygalacturonic acid (PGA) by fungal polygalacturonase (FPG). ○–○, Relative viscosity change; ■–■, difference in the concentration of reducing groups for 1% PGA solution as a function of the FPG concentration. The reaction mixture, containing 0.15 M NaCl in 0.02 M acetate buffer, pH 4.5, was incubated at 40 °C for 30 min.

Table 1
TLC of products obtained by degradation of pectin preparations from apple cv. Budimka (DP 134, DE 95% and 93% PGA)

Reaction time	Pectin degradation products – oligogalactouronides DP 2–8 and monogalactouronic acid							
	Octa-	Hepta-	Hexa-	Penta-	Tetra-	Tri-	Di-	Mono-
$4 \times t_{1/2}$[a]	++	+	/	/	/	/	/	/
$10 \times t_{1/2}$	++	+	+	+	(+)	/	/	/
$20 \times t_{1/2}$	+	++	++	+	+	+	(+)	/
$40 \times t_{1/2}$	(+)	+	++	++	++	++	+	+
$100 \times t_{1/2}$	(+)	(+)	+	++	+++	+++	++	++

Enzymes were FPG (162 U/l) and FPE (27 U/l) from *Aspergillus niger* for different reaction times.
(+) Very weak spot, + weak spot, ++ strong spot, +++ very strong spot.
Reaction conditions: 1% PGA, 0.15 M NaCl, 0.02 M acetate buffer, pH 4.5, 40 °C.
[a] $t_{1/2}$ is time necessary for decrease of the viscosity of the reaction mixture by 50%, determined by TLC as function of whole-number integers of time to the estimated degree of hydrolyzed substrate.

presented in Table 1 also indicates a mode of action of endolytic enzymes called "single attack" (Parenicova, Kester, Benen, & Visser, 2000).

Recent studies of biochemical and kinetic characterization of seven members of the *A. niger* PG-encoding gene family have shown great differences (Benen et al., 1999). For three of the endo-PG (C, D, and E) with the lowest turnover rates, it was supposed that PGA is not their natural substrate, while two constitutive enzymes, PGA and PGB, preferred low DE PGA. New achievements and explanations in the study of PG from *A. niger* were reported regarding its kinetics and mode of action (Parenicova et al., 2000), characterization of the number of subsites in binding to substrate (Benen et al., 1999), and 3-D structure of the enzyme (Van Santen et al., 1999).

3.4. Effect of PG on PE activity

The initial reaction rate of pectin hydrolysis by PE was measured in order to determine whether the PG affects PE activity. The results are presented in Fig. 4. Two parallel plots were obtained for initial velocities when the FPG was supplemented by an additional 15 U/l of PPE. According to the results, it is obvious that the PG expressed no effect on the PE activity. This result could be expected since PG and PE are acting in the cleavage of different bonds in pectin molecules, and naturally they are not competing for the substrate. In contrast to that, there is a very old report from Jansen, MacDonnel, and Jang (1945), about a small contribution of PG to the PE activity on partially esterified PGA. Generally, both PG and PE enzymes contribute to the efficient pectin hydrolysis, and both occur as part of coordinated pectin-degrading machinery in vivo, e.g., in plants. The main role of PG is to depolymerize the pectin, while the role of PE is to hydrolyze the esters present in the pectin backbone. In the case when the substrate is highly methylated, PE enzymes are supposed to help the action of PG, since the activity of PG enzymes is, in general, lower on substrates with high DM (Benen et al., 2003b).

3.5. Effect of PE on pectin degradation by PG

The depolymerization of 1% pectin solution at pH 4.5 in the presence of 0.15 M NaCl was systematically examined with various concentrations of PE and PG. Usually, the amount of FPG was kept constant (162 U/l) and was supplemented with various amounts of PE of fungal (FPE) or tomato plant (PPE) origin. The viscosity of the solutions and methanol content in the reaction mixture are plotted as a function of time in Fig. 5. It can be seen that a lower viscosity decrease was obtained by the supplementation of FPG with PPE (samples 1, 2, and 3) compared to the viscosity decrease obtained by supplementation of FPG with FPE (samples 4, and 5). The amount of the methanol released was lower for the combinations of FPG and PPE (samples 1, 2, and 3) compared to combinations of FPG and FPE (samples 4, and 5). Lower activities of plant esterase obtained may be a consequence of the fact that the reaction was performed at pH 4.5, which is not optimal for PPE (6.5). The other reason may be a different mode of action of plant esterase, which generally removes blocks of methyl groups on a single chain, while fungal esterase attacks the methyl groups on the pectin randomly (Benen et al., 2003c).

In Fig. 6, the effect of the addition of various amounts of FPE to a constant amount of FPG (162 U/l) on the time period required to reach an estimated viscosity ratio η/η_0 of 1.05 is studied. The content of subsequently accumulated MeOH in relation to the concentration of FPE is also

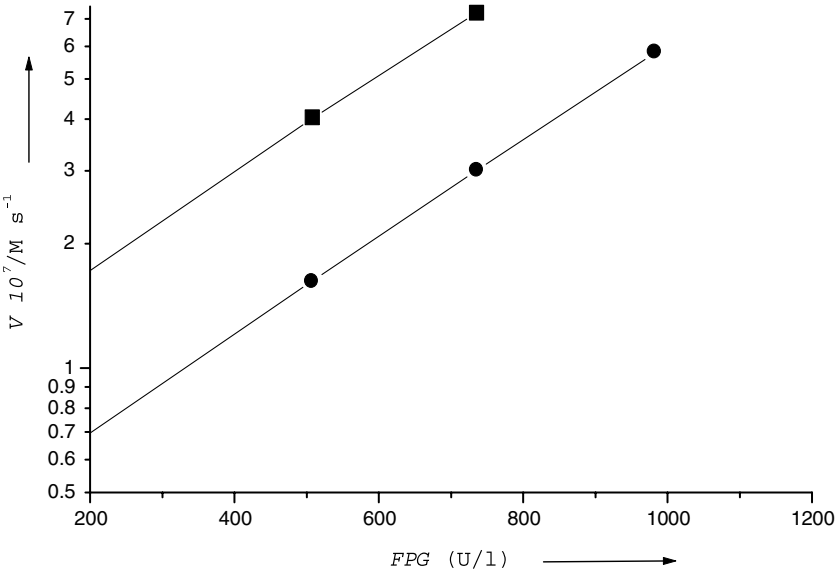

Fig. 4. The effect of FPG on FPE activity. Line ●–● represents PE activity in the FPG preparation. The upper line ■–■ was obtained when the FPG preparation was supplemented by an additional 15 U/l of PPE. Parallel lines indicate that FG does not influence PE activity under the assay conditions. The reaction mixture contained 1% PGA, 0.15 M NaCl, 0.02 M acetate buffer, pH 4.5; 40 °C; 30 min.

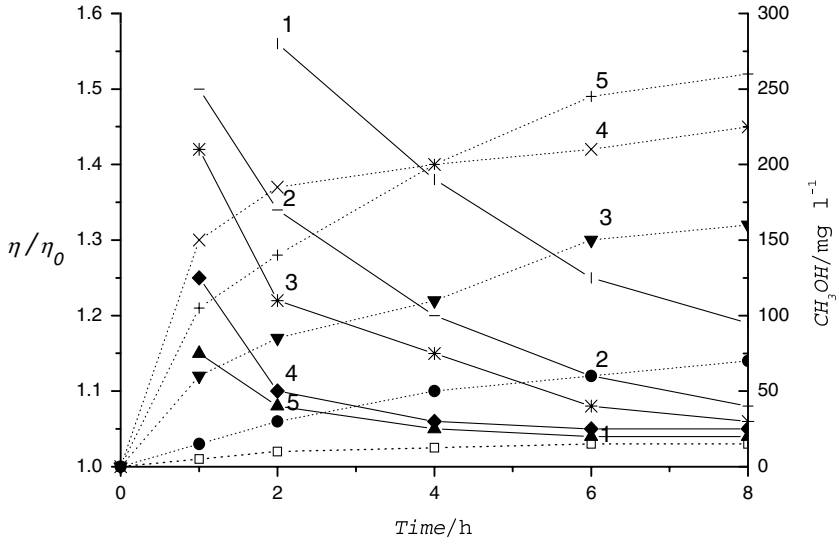

Fig. 5. The effect of PE on PG activity. Right ordinate, MeOH production (---); left ordinate (—), viscosity of the reaction mixture. The reaction mixture contained 1% PGA and 0.15 M NaCl 0.02 M acetate buffer, pH 4.5 and the incubation proceeded at 40 °C, 30 min. The enzymes were used in the following ratios PE:FPG (U/l): (1) PPE:FPG = 15:162; (2) PPE:FPG = 30:162; (3) PPE:FPG = 61.5:162; (4) FPE:FPG = 32:162 and (5) FPE:FPG = 64:323.

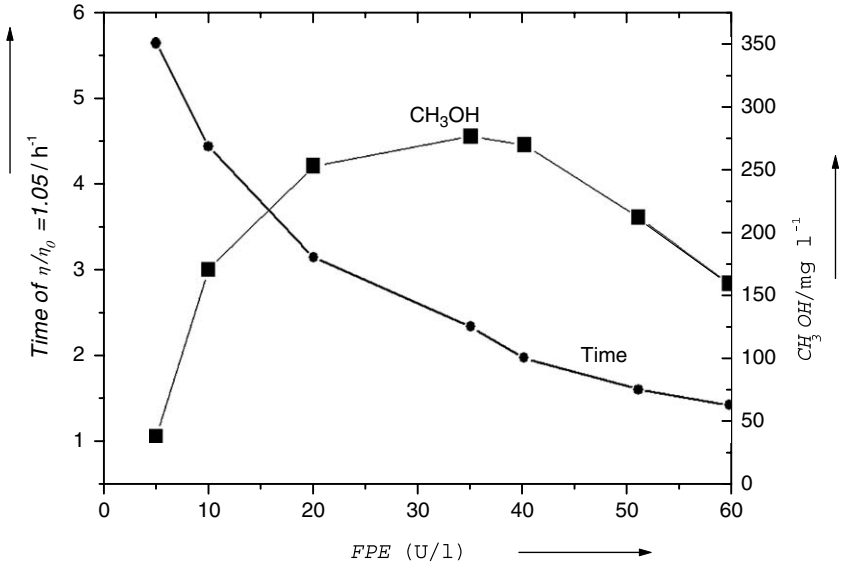

Fig. 6. Effect of addition of FPE to a constant amount of FPG (162 U/l) on the time period necessary to reach a viscosity ratio η/η_0 of 1.05. The right ordinate represents the amount of MeOH accumulated during pectin hydrolysis as a function of the activity of esterase in the solution containing 162 U/l FPG.

presented. It is important to note that the methanol is an undesirable product in juice processing, and could influence the action of PG and PE during the clarification of the juice (Massiot, Perron, Baron, & Drilleau, 1997).

When low amounts of fungal pectin esterase were applied, a longer time was needed to reach the preset degree of pectin degradation. By increasing the pectin esterase concentration the time necessary to reach a viscosity ratio, η/η_0, of 1.05 was shorter, meaning that the reaction could be faster accomplished. As depicted in Fig. 6, the amount of released MeOH increase with the increase of amount of FPE, and is maximal at an enzyme concentration of 35 U/l of the reaction mixture. After that, the amount of released MeOH slightly decreased, suggesting an inhibitory effect. Theoretically, an FPE concentration of 35 U/l will accomplish a complete hydrolysis of the applied concentration of substrate in around 21 h.

Massiot et al. (1997) reported a maximal production of methanol obtained by concerted action of FPG and FPE after more than 2 h of enzymatic reaction, which agree well with our results (Fig. 5). In addition, they found that 65% of the released methanol was due to the action of pectin esterase.

As depicted in Fig. 6, by using the combination of FPG (162 U/l) and FPE (60 U/l), e.g., in a ratio of 2.5, an efficient pectin degradation process with the viscosity

reduction of $\eta/\eta_0 = 1.05$ could be reached in less than 2 h. This process produced about 160 ppm of MeOH in the pectin digest.

3.6. Long-term analysis of hydrolytic products of apple pectin by A. niger PG and PE

The contents of oligogalacturonic acids (DP $\leqslant$ 8) during different stages of pectin hydrolysis are shown in Table 2. The catalytic activity of enzymes from *A. niger* was very low after 12 h of incubation. At this point, the hydrolysate contained mostly trigalacturonic acid. Pectin hydrolysis (DP = 134) was limited to 28.4% of the galacturonic acid bonds. After prolonging the incubation time to 24 h, a slight change in the content of individual oligogalacturonic acids occurred and the final hydrolysate contained 6.84%, 18.6%. 34.1%, 17.2%, 4.42%, of mono-, di-, tri-, tetra-, and pentamer, respectively, and low amounts of other oligomers.

The kinetic profile of oligogalacturonides during different stages of pectin hydrolysis is presented in Fig. 7.

The quantitative composition of the pectin hydrolysates depended on the time of enzymatic reaction. The composition of 12 h-hydrolysate of 93% galacturonic acid DP = 134 to oligomer DP $\leqslant$ 8 is given in Table 2. The degree of pectin hydrolysis was 29.1% (5.07/1 + 16.3/2 + 28.4/3 + 17.5/4 + 6.28/5 + 4.30/6 + 1. 26/7). This was in accordance with the degree of pectin hydrolysis obtained by determining the reducing groups. Theoretically, the maximal hydrolysis of the substrate should be 33.3%, if trimers represent the only end products. If monomers or dimers are the only end products of pectin hydrolysis, the maximal degree of hydrolysis should be 100% and 50%, respectively. Our result for hydrolytic pectin degradation of 30.1% shows that the average degree of polymerization of end products is 3.36 (100/29.8). The results agree with the results obtained by McClendon (1975). This author utilized yeast endo-polygalacturonase, pectinol 45AP and

Table 2
Contents of oligogalacturonic acids (DP 2–8) in hydrolysate of Budimka apple pectin preparations (DP 134, DE 95%, 93% PGA) by combined catalytic activity of FPG (162 U/l) and FPE (27 U/l)

Oligomer	Oligogalacturonic acids% (w/w) at different time intervals									
	15 (min)	30 (min)	60 (min)	90 (min)	120 (min)	150 (min)	180 (min)	240 (min)	12 (h)	24 (h)
1	0	0	0	0.27	0.68	0.82	0.87	1.32	5.07	6.84
2	0	0	0.74	1.58	2.28	3.17	3.58	4.69	16.3	18.6
3	0.75	0.98	1.32	2.18	4.36	9.6	13.48	16.4	28.4	34.1
4	0.47	0.35	0.63	2.4	6.48	7.82	9.16	14.6	17.5	17.2
5	0.69	0.47	0.52	3.84	7.94	9.27	11.2	11.4	6.16	4.42
6	1.36	1.6	3.92	7.65	12.7	10.6	11.7	6.19	5.8	0
7	2.06	6.84	12.4	24.1	15.3	12.7	8.98	5.04	2.26	0
8	12.6	15.3	19.3	25.1	23.1	21.5	14.5	9.31	0	0
DP $\leqslant$ 8	17.9	25.6	38.8	67.1	72.9	75.5	73.5	68.8	81.5	81.1

Reaction conditions: 1% PGA, 0.15 M NaCl, 0.02 M acetate buffer, pH 4.5, 40 °C.

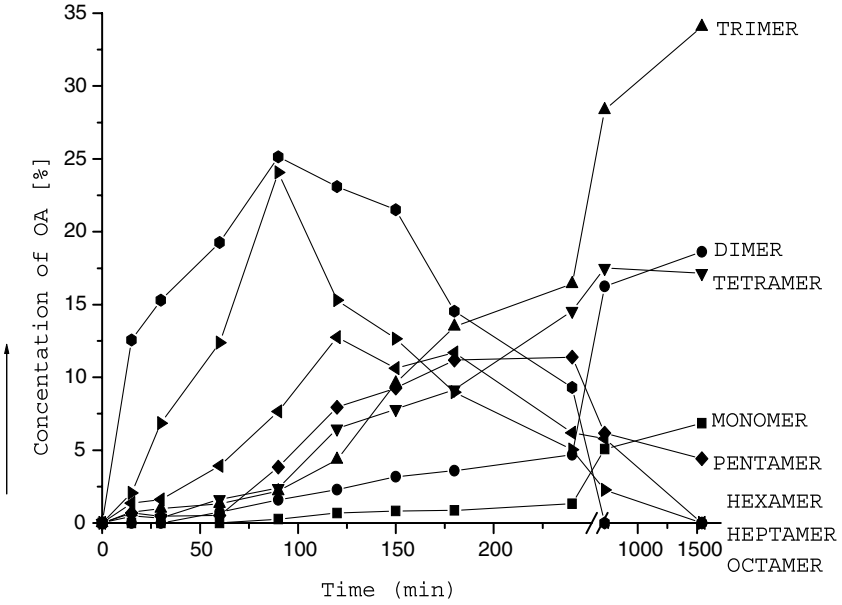

Fig. 7. Effect of time on the concentration of oligogalactouronic acid (OA) of 1% PGA from Budimka apple hydrolyzed at 40 °C by combined catalytic activity of FPG (162 U/l) and FPE (27 U/l).

pectinol 46AP (both from *Aspergillus*) and reported 25%, 32% and 38% hydrolysis, respectively, at ambient temperature and pH 5.5.

4. Conclusion

Enzymatic degradation of apple pectin derived from our autochthons Budimka apple variety (*P. malus* L.) was studied by individual and/or combined action of fungal polygalacturonase from *A. niger* (FPG), fungal pectin esterase from *A. niger* (FPE) and plant tomato pectin esterase (PPE). The process conditions, such as time, temperature, pH and the levels of the released MeOH were studied in order to attain an appropriate chemical composition and technological properties of the products during fruit processing.

The optimum pH values for FPG, FPE and PPE were determined to be 4.5, 3.5 and 6.5, and optimum temperatures were 40, 45 and in a range from 45 to 50 °C, respectively. The action of PE did not affect the action of PG used in this study. However, the PE contributed to more efficient substrate degradation. FPE was found to be more efficient for hydrolysis of the apple pectin than was PPE from tomato in combination with FPG. By measuring initial velocities, on 1% apple pectin, it was confirmed that the PG expressed no effect on the PE activity. By using the combination of FPG (162 U/l) and FPE (60 U/l), e.g., in a ratio of 2.5, an efficient pectin degradation process with the viscosity reduction of $\eta/\eta_0 = 1.05$ could be reached in less than 2 h. This process produced about 160 ppm of MeOH in the pectin digest.

The hydrolysis reaction with FPG (162 U/l) and FPE (27 U/l) achieved a degree of hydrolysis of around 29% after 12 h and the product consisted mostly of trimers (28.4%).

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