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Separation of pectin methylesterases and polygalacturonases on monolithic columns ☆

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Abstract

The most abundant isoforms of tomato pectin methylesterase (PME; EC 3.1.1.11; M_r 26 kDa), polygalacturonase (PG; EC 3.2.1.15; PG1 with M_r 82 kDa) and a basic protein with M_r 42 kDa and unknown function were isolated from fresh tomato fruit by a fast chromatographic procedure on a Convective Interaction Media (CIM®) short monolithic disk column bearing carboxymethyl (CM) groups. The extraction of the targeted enzymes with 1.2 M NaCl solution was followed by precipitation with ammonium sulfate at 60% of saturation, solubilisation of the pellet in 0.5 M NaCl and fractionation using a linear gradient from 0 to 700 mM NaCl. Among six fractions five had PME activity and four had PG activity, while one fraction containing a pure protein with M_r 42 kDa with neither of these activities. Two concentrated fractions, one with PG and one with PME were further purified. A linear gradient from 0 to 500 mM NaCl with 20% CH_3CN in the mobile phase was used for the PG fraction and two CM disks and a linear gradient from 0 to 200 mM NaCl were used for the PME fraction as a greater capacity was necessary in this case. From 4 kg of fresh tomato flesh we obtained 22 mg of purified PME, 1.8 mg of purified, active PG1, 13.5 mg of additional basic protein and a fraction with PG2 contaminated by a PME isoform. Carboxymethyl CIM disk short monolithic columns are convenient for semi-preparative and analytical work with tomato fruit pectolytic enzymes.
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1. Introduction

Pectins and cellulose microfibrils are the two major polysaccharide networks in the plant cell wall. Pectins (polygalacturonans, rhamnogalacturonans) have complex structures but the majority of each structure consists of homopolymeric poly-α-(1 → 4)-D-galacturonic acid residues, partially present as their methyl esters. The properties of pectins depend on the degree of esterification, which is normally about 70%. The controlled de-esterification, converting high methoxyl pectins to low-methoxyl pectins, is possible using pectin methylesterases (PME, EC 3.1.1.11), whereas for the degradation of the polygalacturonan backbone polygalacturonase (PG, EC 3.2.1.15) is needed. Both types of enzymes and their different forms were found in different plant sources in last few decades and have been isolated and studied. Some additional known enzymes such as pectate lyase, β-galactosidase, arabinosidase, β-mannosidase and endo-β-mannanase [1,2] as well as some unknown enzymes [3] may also be involved in pectin transformations, because pectins can be covalently bonded to some other polysaccharides, such as glucomannans and galactomannans, present in the plant cell wall [2]. PME and PG, the most studied cell wall enzymes, were also found in fungi, some bacteria and yeasts although with different characteristics (e.g. optimal pH value for the highest activity), and they play an important role in the pathogenecity of some of these organisms [4,5]. Some microorganisms serve as the source of pectolytic enzymes for industrial applications [6–8].

Natural tomato (*Lycopersicon esculentum*) fruit is a rich source of several PME isomorfs [9,10] and at least three forms of endo-polygalacturonase (PG1, PG2a and PG2b), all containing the same polypeptide derived from a single gene [11]. Presence of an exo-PG, an enzyme which can cleave galacturonic acid only at the end of the polygalacturonic acid macromolecule, has also been reported in tomato fruit [12]. Transgenic tomato fruits

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with reduced PG [13] or PG and PME activity [14] were produced and studied. It was found that PG is not the only enzyme responsible for the fruit softening during ripening; only a part of a series of processes leading to the breakdown and softening of fruit tissues is catalyzed by PG [15].

PME and/or PG were isolated from tomato fruits by different procedures, which all include ion-exchange chromatography followed by several additional steps such as ion-exchange on DEAE – cellulose followed by affinity chromatography on Heparin Sepharose [16], and four chromatographies – ion-exchange on DEAE-Sephadex A-50, ion-exchange on S-Sepharose, gel filtration on Sephadex G-75 and final ion-exchange on Mono S [35]. Our recently published procedure for isolation of PME is the only one performed using a monolithic stationary phase and provides a much faster and more efficient way to isolate PME [17]. A summary of the preparation, properties and applications of monolithic materials, the newest generation of stationary phases for liquid chromatography, exploiting different principles such as ion-exchange, affinity recognition, reversed-phase and hydrophobic interaction was recently published [18]. Monoliths have been widely used for preparative and analytical separation of biopolymers during the last decade [19,20]. One of the first useful monolithic stationary phases for the rapid separation of proteins was designed in a disc format [21] and is the basis of short bed Convective Interaction Media (CIM®) disks monolithic columns, which are specific among the chromatographic columns, because of their monolithic structure and extremely short column length (3 mm). They have very fast mass transfer between the mobile and stationary phase, which provides high speed and high efficiency of the separation [18]. CIM disk monolithic columns have been used successfully in the separation of low and high molecular mass substances [22,23], proteins [24,25], separation and purification of plasmid and genomic DNA [26–28] and purification of plant and microbial enzymes [17,29]. They have also been used for the direct synthesis of peptides [30,31] and as enzyme reactors [32–34].

Recently, we reported the isolation of pure PME from tomato fruits by means of two cation-exchange chromatographies on short monolithic columns based on polymethacrylates [17]. Although the enzyme extraction was performed with dilute HCl at pH 1.6 according to Pressey [35] we found in addition to the targeted PME activity some PG activity in one of the chromatographic fractions.

The aim of the work described here was the isolation of the most abundant isoform of pure PME and one form of PG enzyme from the same NaCl extract of tomato fruit with the minimum number of chromatographic steps using the same type of short monolithic columns. Some attention has been devoted also to additional proteins appearing in the chromatographic fractionation of tomato extract preparation.

2. Experimental

2.1. Chemicals

MW-SDS-70L (kit for molecular weights 14.000–70.000), Tris-(hydroxymethyl)amino-methane (Tris), bicinchoninic acid (BCA) kit for protein determination and 3,5-dinitrosalicylic acid were purchased from Sigma–Aldrich (Steinheim, Germany). Sodium hydroxide, sodium chloride, sodium acetate, acetic acid, ammonium sulfate, ethanol and acetonitrile were from Merck (Darmstadt, Germany). Polygalacturonic acid and pectin with 70–75% degree of esterification from apples were obtained from Fluka Chemie (Buchs, Switzerland). Phenol red was purchased from Coleman & Bell (Norwood, OH, USA) and potassium sodium tartrate tetrahydrate from Kemika (Zagreb, Croatia). All the solutions were prepared with deionized water (Millipore Milli-Q).

2.2. Extraction of PG and PME

Ripe tomato fruits were purchased from the local market and the extraction was performed at 4 °C. After removal of the peels and the seeds the tomato flesh (500 g) was homogenized. After the addition of 500 ml of cold water and homogenisation with an Ultraturrax (rotor/stator homogeniser, Ultraturrax/Ika T 25) the pH of the homogenate was adjusted to 3 with 1 M HCl then the solution was mixed for 5 min by the Ultraturrax. The pellet obtained after centrifugation for 20 min at 8000 rpm was dissolved in 750 ml of water using Ultraturrax. After centrifugation of the solution at 8000 rpm for 20 min, the pellet was dissolved in 500 ml of 1.2 M NaCl (pH 6) using Ultraturrax for 1 min then stirred for 3 h with a magnetic stirrer. After centrifugation for 20 min at 10,000 rpm the supernatant was filtered through a filter paper and a membrane filter [Millipore Millex – HV hydrophilic poly(vinyldiene difluoride) – PVDF 0.45 μm]. Ground ammonium sulfate was added to the supernatant to achieve a saturation of 60%. After shaking for 5 min and centrifugation for 20 min at 10,000 rpm the pellet was dissolved in 40 ml of 0.5 M NaCl and the solution filtered through 0.45 μm filters. Ultrafiltration (Amicon, 8400) was performed using an XM50 membrane (DIAFLO ultrafiltration membranes, Amicon Corporation, Danvers, MA, USA). Sodium chloride present in the XM50 concentrate was removed by passing this solution through a PD-10 column (prepacked Sephadex G-25 column, Amersham Biosciences, England). A PD10 column was equilibrated with 5 ml × 2.5 ml 20 mM Tris (2.42 g Tris base per l neutralised with concentrated HCl 10 times diluted to pH 7.4). Concentrate XM50 (2.5 ml) was desalted by elution with 3.5 ml 20 mM Tris, pH 7.4 and the first 3.5 ml were collected and filtered through 0.45 and 0.20 μm membrane filters (Minisart®, Sartorius) before the chromatographic fractionation. The solutions were stored at +4 °C.

2.3. Determination of protein content

The concentrations of proteins in the extract and in the HPLC fractions were determined by a spectrophotometric Micro BCA Protein Assay (Sigma) using a bicinchoninic acid kit.

2.4. HPLC fractionation and purification

The HPLC system consisted of a pump ConstaMetric 4100 (Thermo Separation Product—TSP, Riviera Beach, CA, USA),

autosampler AS3000 (TSP) with a fixed 200 μl loop and UV detector SpectroMonitor 3200 (TSP) set to 280 nm. Separation was performed on Convective Interaction Media disk monolithic columns (diameter 12 mm, length 3 mm) bearing carboxymethyl (CM) cation groups from BIA Separations, Ljubljana, Slovenia. The CIM disk monolithic columns could be regenerated by placing them into 1 M NaOH water solution.

Fractionation of the PME and PG isoenzymes from desalted XM50 concentrates of tomato extract was performed with a linear gradient of sodium chloride using a mobile phase consisting of buffer A: 20 mM Tris (2.42 g Tris base per 1 l neutralised with concentrated HCl diluted 10 times to pH 7.4) and buffer B: buffer A containing 1 M NaCl, pH adjusted to pH 7.4. The gradient program for the fractionation at a flow rate of 4 ml/min was as follows: linear gradient 100% to 30% A (3 min), 30% A to 100% A (0.1 min), 100% A (2.9 min).

Collected fractions with PME or PG were concentrated by ultrafiltration using YM10 (Millipore Corporation, Bedford, USA) or YM30 (DIAFLO ultrafiltration membranes, Amicon Corporation), respectively. The concentrates obtained were desalted with a PD-10 column and the eluates filtered through 0.45 and 0.20 μm membrane filters (Minisart, Sartorius) before the next chromatographic step.

The PME YM10 concentrate was further purified on a column consisting of two CIM CM disks in the same housing, using the same mobile phase and the flow rate used in the fractionation. The following gradient was used: linear gradient 100% to 80% A (3 min), 80% A to 100% A (0.2 min), 100% A (0.8 min). The purified PME fraction was concentrated 10–15 fold by ultrafiltration using a cut-off YM10.

The PG YM30 concentrate was further purified with the same column and the flow rate used for fractionation. The mobile phase consisted of buffer A: 20 mM Tris (2.42 g Tris base per 1 l neutralised with concentrated HCl diluted 10 times to pH 7.4) mixed with acetonitrile 4:1 (v/v) and buffer B: buffer A and 1 M NaCl mixed with acetonitrile 4:1 (v/v). The following gradient was used: linear gradient 100% to 50% A (6 min), 50% to 100% A (0.1 min). To remove acetonitrile, the obtained PG1 fraction and the fraction of PG2 contaminated by PME were subjected to rotary vacuum evaporation at 35 °C until no smell of acetonitrile remained and finally, the pure PG1 fraction and the contaminated PG2 fraction were concentrated by ultrafiltration using a cut-off XM50.

2.5. PME activity assay

Substrate: pectin (0.4 g) was dissolved in 80 ml of water while heating. After cooling, 1.17 g NaCl and 24.2 mg Tris and 2 ml of indicator solution (9 mg phenol red dissolved in 20 ml 20% (v/v) ethanol/water) were added and the pH adjusted to 7.5 (raspberry red colour) with 0.1 M NaOH or 0.1 M HCl then water was added to a total volume of 100 ml. For visual estimation of the activity 50 μl of the test solution (pH 7.5) is mixed with 0.5 ml of the substrate; a change in colour from red to yellow indicates activity.

2.6. PG activity assay

The PG activity was measured as the amount of galacturonic acid released from polygalacturonic acid under defined conditions. The spectrophotometric reagent used for galacturonic acid was 3,5-dinitrosalicylic acid.

Extraction buffer: (A) acetic acid (1.1 ml) was pipetted into 100 ml volumetric flask which was then filled with water (0.2 mol/l). (B) 1.641 g of sodium acetate was dissolved in water and the solution made up to 100 ml (0.2 mol/l). An 80 mM buffer solution is prepared by mixing 11.8 ml of A and 28.2 ml of B in a 100 ml volumetric flask, adding water to 100 ml, and adjusting the pH to 5.0.

Substrate: 0.25% polygalacturonic acid in the extraction buffer.

Detection reagent: K–Na-tartrate tetrahydrate (30 g) was dissolved in water, 20 ml of 2 M NaOH was added and the solution made up to 100 ml with water. 3,5-Dinitrosalicylic acid (25 mg) was dissolved in 25 ml of this solution.

The sample test solution (0.75 ml) containing about 0.2 mol/l NaCl was mixed with 0.75 ml of the substrate and the mixture was incubated at 37 °C for 30 min (pipet every 30 s). The enzyme reaction was stopped by adding 1.5 ml of the detection reagent and incubating the mixture in boiling water for 10 min. It was then cooled in cold water and the absorbance at 540 nm was read against filtered reference prepared without enzyme. Turbid yellow colored samples (low PG activity) were filtered before measurements. Samples with high PG activity give orange–brown colour.

2.7. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The purity and molecular masses of PME and PG were determined by SDS-PAGE on slab gel prepared with 15% (resolving gel) and 4% (stacking gel) acrylamide by the method of Laemmli [36]. The samples of tomato crude extract, fractions 1–6 and final purified PME and PG1 fractions were applied in parallel with protein standard marker MW-SDS-70L (kit for molecular weights 14.000–70.000). After electrophoresis, the gels were subject to silver staining according to Heukeshoven and Dernick [37].

3. Results and discussion

Recently, we used diluted HCl at pH 1.6 for the extraction and isolation of PME from tomato fruit [17]. Fractionation of the extract on CIM sulfonyl (SO_3) disk monolithic column gave a minor fraction with PME and PG activity. To improve the yield of PG, we decided to perform another extraction procedure according to Pressey [38]. This procedure, in which the enzymes are solubilised with 1.2 M NaCl at pH 6.0, has also been used by other authors [39,40]. In order to optimise the procedure, we studied the effect of precipitation at 30–80% ammonium sulfate saturation. Similar results for protein content and PME and PG activity were obtained with 50–80% of ammonium sulfate saturation. Significantly lower amounts of PG were obtained with 30 and 40% saturation (Fig. 1). Subsequently, the targeted enzymes

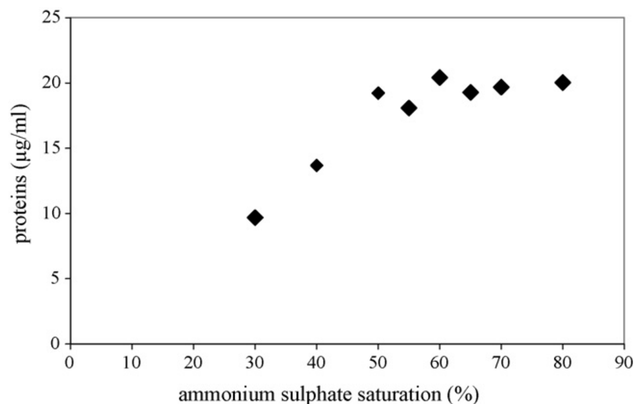

Fig. 1. The effect of precipitation at 30–80% ammonium sulfate saturation.

were separated from the remaining proteins by precipitation with 60% ammonium sulfate saturation and the pellet that was finally obtained was dissolved in 0.5 M NaCl.

In view of our previous work and published data concerning the molecular masses of PME and PG2, it was decided to purify the solution further using ultrafiltration by cut-off XM50 and then concentrate it by cut-off YM10. After the first ultrafiltration we expected to get the targeted enzymes PME and PG2 in the XM50 filtrate (separation from proteins with higher molecular masses) but this was not the case. Only the XM50 concentrate was shown to have PME and PG activity. Before the chromatographic fractionation, the XM50 concentrate was desalted with a PD10 column and filtered through 0.45 and 0.20 μm membrane filters. PME and PG were separated from the remaining proteins by cation-exchange chromatography using a CIM CM disk monolithic column with Tris buffer (20 mM, pH 7.4) and sodium chloride in a linear gradient from 0 to 700 mM. The chromatogram obtained from the fractionation of the proteins is shown in Fig. 2. Compared to our previous fractionation [17], in which we used a CIM SO_3 disk, and the same linear gradient obtaining just three peaks (three fractions with PME and one with PG activity), better selectivity was achieved on the CM disk. All fractions indicated by the chromatographic peaks 1–6 in Fig. 2 were collected from one chromatographic run and were monitored for protein content and PME and PG activity and for purity by SDS-PAGE (silver staining, Fig. 3).

Determination of PME and PG activity showed there to be PME activity in all the fractions except fraction 2 and PG activity in all fractions except fractions 2 and 3. Additionally, a colour change from raspberry red to yellow was observed during the PME activity testing of fraction 2. However, this was 15 min after the beginning of the assay, at which time the blank remained unchanged. In the case of all the other tested fractions the colour change happened immediately. Therefore, it is not clear whether this activity is due to contamination, which was not detected on the SDS-PAGE, or is the consequence of the activity of this protein. As we have already reported for the PME activity assay [17] it is essential to mix (vortex) the sample solutions just before taking an aliquot for the protein assay and the PG activity assay. The purity of the isolated fractions 1–6 and the tomato extract were checked by SDS-PAGE silver staining (Fig. 3) and it was

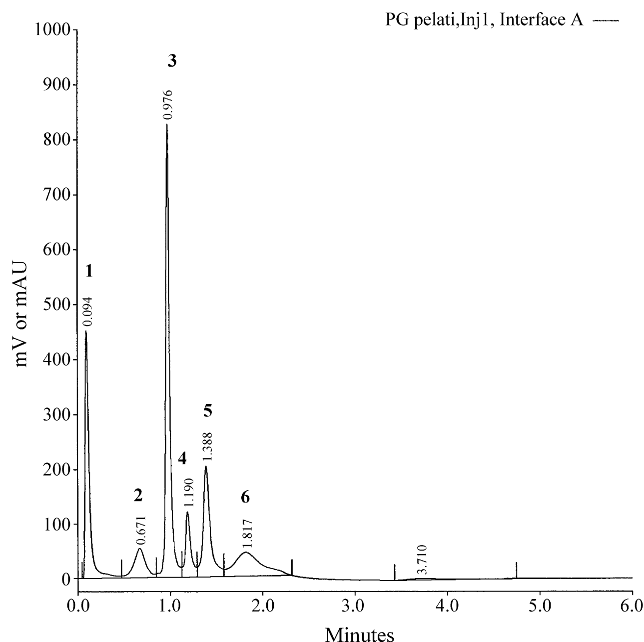

Fig. 2. Fractionation of XM50 concentrate of tomato extract on CIM CM disk monolithic column (diameter 12 mm, length 3 mm) using linear gradient elution and an injection volume of 200 μl. PME activity was detected in fractions 1 and 3–6, while PG activity was detected in fractions 1 and 4–6. Peak 2 failed to show PME or PG activity. Binding buffer (A): 20 mM Tris, pH 7.4, elution buffer (B): buffer A containing 1 M NaCl, pH 7.4; flow rate 4 ml/min; gradient: linear gradient 0% to 70% B (3 min), 30% A to 100% A (0.1 min), 100% A (2.9 min).

shown that fraction 2 was a pure protein. After concentration of this fraction by ultrafiltration using cut-off YM30 we obtained 13.5 mg of this basic protein which has molecular mass 42 kDa as determined by SDS-PAGE. However, its function and activity are unknown. It cannot be β-subunit (the protein which complexes with PG2 to form PG1), because β-subunit, according to the literature, is an acidic protein with molecular mass 38 kDa and is resolved poorly on SDS gel [41,42].

PME activity independent of the presence of NaCl in the substrate [10,17] is found only in fraction 3. The SDS-PAGE for this

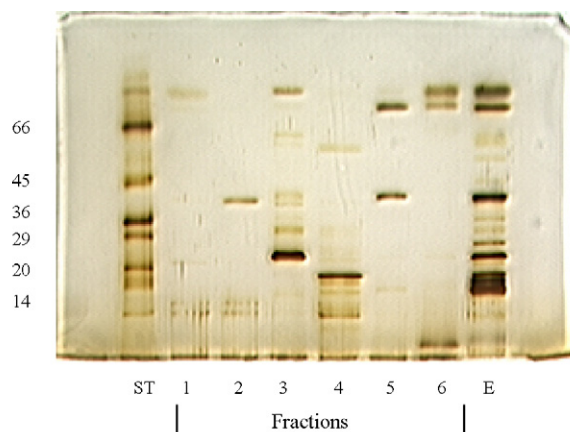

Fig. 3. 15% SDS-PAGE with silver staining for purity control of fractions obtained after fractionation of XM50 concentrate of tomato extract on CIM CM disk monolithic column (Fig. 2). ST: standard protein marker (values in $M_r \times 10^3$ at left-hand side); 1–6: collected fractions; E: tomato crude extract.

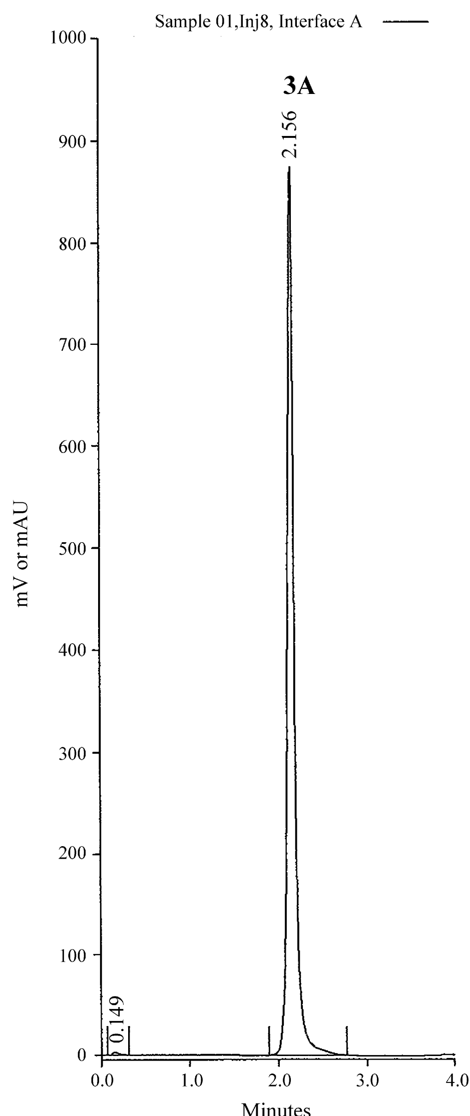

Fig. 4. Purification of PME isoenzyme from the YM10 concentrate of fraction 3 (Fig. 2) on a CIM CM column (two disks with diameter 12 mm and length 3 mm) using linear gradient and an injection volume of 200 μl. Binding buffer (A): 20 mM Tris, pH 7.4, elution buffer (B): buffer A containing 1 M NaCl, pH 7.4; flow rate 4 ml/min; gradient: linear gradient 100% to 80% A (3 min), 80% A to 100% A (0.2 min), 100% A (0.8 min). Fraction 3A showed "salt independent" PME activity.

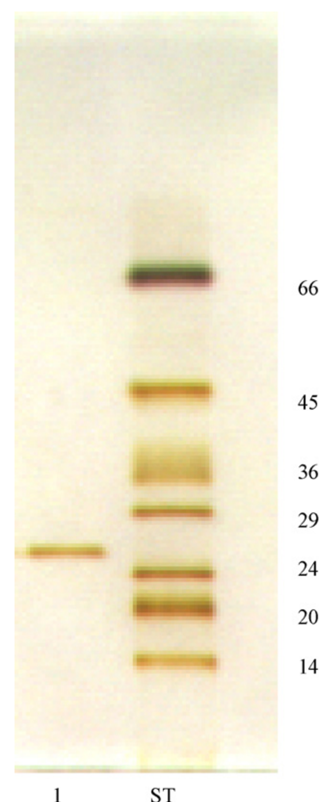

Fig. 5. Fifteen percent SDS-PAGE with silver staining for purity control of the targeted PME—fraction 3A (Fig. 4) purified from fraction 3 (Fig. 2) on CIM CM disk monolithic column. Lane 1: targeted PME—fraction 3A; ST: standard protein marker (values in $M_r \times 10^3$ at right-hand side).

fraction showed a band with the same molecular mass (26 kDa) as has been described [17] and it can be concluded that this is the same PME isoform that was already isolated in pure form using the second chromatographic step on the same monolithic column and mobile phase, but with the step gradient of NaCl. Fraction 3 was concentrated by means of ultrafiltration using cut-off YM10 in order to separate the PME band (Fig. 3, lane 3) from the upper bands. The YM10 concentrate was desalted via PD10 column and filtered through 0.45 and 0.20 μm membrane filters. The targeted PME was further purified by using two CIM CM disks in the holder (to increase the capacity of the column) and a linear gradient from 0 to 200 mM NaCl (Fig. 4). The PME fraction was concentrated using cut-off YM10. Purity testing of the isolated PME by SDS-PAGE (Fig. 5, lane 1) showed that the last chromatographic step was successful. The yield of purified PME isoenzyme was 22 mg from 4 kg of tomato flesh.

Among the fractions with PG activity, fraction 5 (Fig. 3, lane 5) was chosen for further purification. It was concentrated by cut-off YM30 and filtered through 0.45 and 0.20 μm membrane filters. Several experiments, varying organic modifiers, buffers, pH were performed to find the proper mobile phase and gradient for the next chromatographic step. Among the tested combinations, acetonitrile proved to be most effective for the separation of PG1 and PG2. In this case cation-exchange is clearly not the only means of separation. Finally, fraction 5 was purified using linear gradient elution from 0 to 500 mM NaCl, 20 mM Tris, pH 7.4 with 20% acetonitrile in the mobile phase.

This resulted in two main fractions (5B and 5C) both with PG activity and one minor fraction (5A) with only PME activity (Fig. 6). After evaporation of acetonitrile the collected fractions were concentrated using cut-off XM50. The fraction with retention time 2.9 min (Fig. 6) showed PG and PME activity, while that with retention time 3.8 min showed only PG activity. As shown by SDS-PAGE (Fig. 7) the PG fraction with the shorter retention time (5B) contained, beside PG2 (about 43 kDa), two other proteins (one in minor quantities), while the PG fraction with the longer retention time (Fig. 7, lanes 5, 6 and 7) was pure PG1. The fractions in lanes 3 and 6 originated from purification of the PG fraction obtained after our previous fractionation of tomato extract on CIM SO_3 disk monolithic column. We

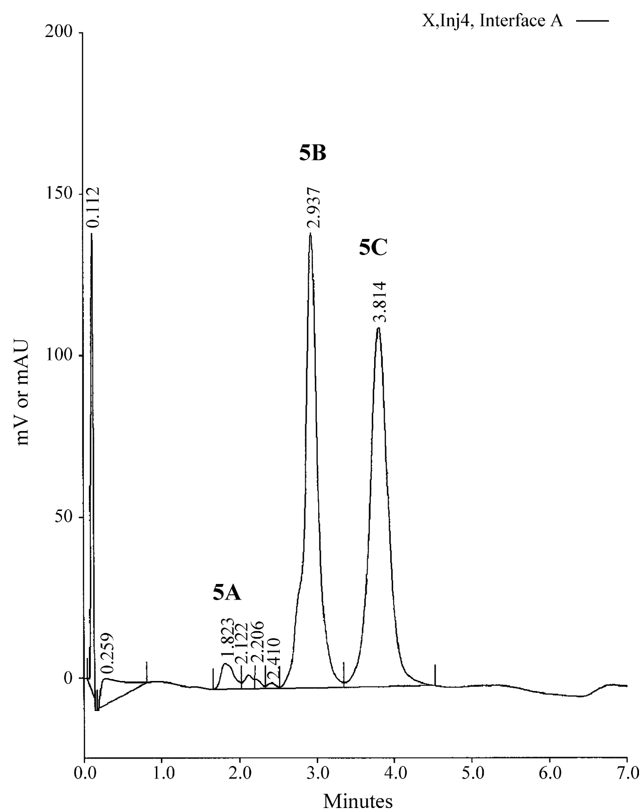

Fig. 6. Purification of PG from the YM30 concentrate of fraction 5 (Fig. 2) on a CIM CM column using linear gradient elution and an injection volume of 200 μl. Binding buffer (A): 20 mM Tris, pH 7.4, 20% acetonitrile; elution buffer (B): buffer A containing 1 M NaCl, pH 7.4; flow rate 4 ml/min; gradient: linear gradient 100% to 50% A (6 min), 50% to 100% A (0.1 min). Fraction 5A showed PME activity, fraction 5B showed PME and PG activity, while fraction 5C showed only PG activity.

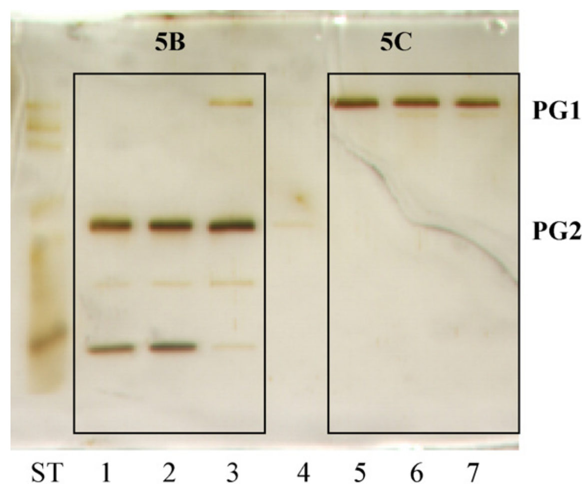

Fig. 7. Fifteen percent SDS-PAGE with silver staining for purity control of the XM50 concentrates of PG fractions 5B and 5C (Fig. 6) purified from YM30 concentrate of fraction 5 (Fig. 2) on a CIM CM disk monolithic column. ST: standard protein marker; lanes 1–3: fraction 5B with PG2 contaminated with PME; lanes 5–7: targeted PG1—fraction 5C. Fractions in lanes 3 and 6 originated from purification of PG fraction obtained after fractionation of tomato extract on CIM SO_3 disk monolithic column [17].

obtained 1.8 mg of PG1 from 4 kg of fresh tomato flesh. The procedure provides the isolation of pure PG1, but not PG2. On SDS-PAGE we have consistently obtained just one band for PG2, not two separate bands for PG2A and PG2B. There is discussion in the literature about the presence of PG1 in tomato fruit. It is not clear, however, if it is produced from PG2 and β-subunit during the extraction or if it is already present in the tomato fruit [43].

During the HPLC, peak distortion appeared occasionally. This problem was solved by simple regeneration of the CIM CM disk monolithic columns, accomplished by removing them from the housing, putting them into 1 M aqueous NaOH solution for at least 1 h at room temperature. Even several weeks of storage in the same solution in the refrigerator had no deleterious effect on the CM disks. Before the chromatographic separation the regenerated disk is first washed with water, then inserted into the housing, attached to the HPLC system, and washed with mobile phase for at least two runs in the case of fractionation and purification of PME, at least three runs being necessary in the case of purification of PG, where the mobile phase contains acetonitrile.

Isolation of pure enzymes from biological matrices is a challenging task but pure tomato PME and PG are readily accessible with these relatively new chromatographic materials that we have used. Our new procedure using CIM CM short monolithic disk columns with two cation-exchange chromatographic steps provides a way of isolating PME and PG that is much faster and more efficient than the published procedures [9,41,44]. Additionally, the BIA Separations method transfer calculator, based upon a formula derived from the transfer of gradient methods for protein separation between columns of different sizes [28], can facilitate a simple scaling-up to the CIM® tube monolithic columns for preparative fractionation of bigger amounts of the enzymes at once than it was in our case (about 200 μg of proteins loaded for one run). Work with smaller amounts of enzymes is also possible using smaller CIM disks than were used in our work.

Acknowledgements

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