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Journal of Chromatography A, 1065 (2005) 121–128
JOURNAL OF CHROMATOGRAPHY A
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Separation of pectin methylesterase isoenzymes from tomato fruits using short monolithic columns

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Available online 24 December 2004

Abstract

One of the main forms of tomato pectin methylesterase (PME; EC 3.1.1.11) that is applicable to the food industry was isolated from fresh tomato fruit. The extraction of the PME isoenzymes involved washing the fresh tomato flesh with water in order to remove sugars and than solubilizing the enzymes with a diluted HCl solution at pH 1.6. The extract was then neutralized to pH 7.4 using buffer solution. After filtration, the solution was directly fractioned using Convective Interaction Media (CIM®) short monolithic disk column bearing sulfonyl (SO_3) groups and using a linear gradient from 0 to 700 mM NaCl. The injection volume was 3 ml and the diameter of the column was 12 mm and length 3 mm. The isolated fractions were monitored for protein content and PME activity. The fraction with the targeted enzyme, which showed NaCl independent activity, was further purified and concentrated by ultrafiltration and finally purified by a second semi-preparative cation-exchange chromatography step using a CIM carboxymethyl (CM) disk monolithic column consisting of two disks and applying a step gradient. From 1 kg of fresh tomato fruits, 7.5 mg of purified PME with molecular mass estimated to be 26 000 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was obtained. A fraction with mixed PME and polygalacturonase activity was also obtained. Compared to the published procedures for the isolation and purification of PME from plant materials, this new procedure is much faster and more efficient. The potential application of CIM disk short monolithic columns in the analysis and semi-preparative extraction and isolation of the PME isoenzyme is presented.

Keywords: Cation-exchange chromatography; Monolithic columns; Pectin methylesterase; Polygalacturonase; Tomato; *Lycopersicon esculentum*

1. Introduction

Pectin methylesterase (PME; EC 3.1.1.11) is plant cell wall enzyme that has also been found in pathogenic fungi and bacteria. PME catalyzes the removal of methyl groups from the polygalacturonic acid chain of pectin. De-esterified pectins are susceptible to the subsequent action of polygalacturonase (PG; EC 3.2.1.15) and pectin lyase (PL; EC 4.2.2.10). However, the role of PME in plant growth and development is not yet understood. Transgenic tomatoes showing a 10-fold reduction in PME activity were used to investigate the role of PME in tomato fruit ripening. The reduced PME activity caused an almost complete loss of tissue integrity during fruit senescence, but showed only a small affect on fruit firmness during ripening [1]. De-esterification of pectins in different parts of the tomato plant or at different times in plant development may involve different forms of PME [2].

The control of PME and related enzymes in food industry is important for improving the quality of food. For example, unmethoxylated pectin is being used in the jam industry as gelling agent. It is able to form gels in the presence of Ca^{2+} ions. However, its substitution with pure PME (uncontaminated with polygalacturonases and pectin lyases) is expected to improve the quality of the product. PME may also be beneficial in the juice industry for the removal of soluble pectin polymers which give unwanted haze formation in the juice [3]. But, the residual activity of the thermostable PME is sometimes undesired, because it is responsible for cloud

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doi:10.1016/j.chroma.2004.12.006

destabilization of fruit juices [4]. The PME inhibitor purified from kiwi fruit can be utilized for the detection of residual PME activity in fruit products and also in PME inactivation [5].

A rich source of PME is tomato (*Lycopersicon esculentum*) fruit which contains three main forms of PME which are all basic proteins with molecular masses between 23.8 and 42 kDa [2,6–8]. Isolation of PME isoforms from tomato fruit extracts [6,7,9] was performed using different chromatographic methods. Ion-exchange chromatography, which is common to all of these published procedures, can now be performed on monolithic stationary phases, which may provide much faster separations then traditional media.

A very useful summary of monolithic materials (preparation, properties and applications) was recently published [10]. In the last decade, monoliths have been widely used for preparative and analytical separation of biopolymers [11,12]. One of the first useful monolithic stationary phases for the rapid separation of proteins was designed in a disc format [13]. Short bed Convective Interaction Media (CIM®) disks monolithic columns are unique among chromatographic columns, because of their monolithic structure and extremely short column length (3 mm). Due to the monolithic structure, significantly enhanced mass transfer between the mobile and stationary phase results in extremely fast separation of large molecules like proteins and DNA [14]. CIM disk monolithic columns are successfully used for the separation of peptides [15], proteins and nucleic acids [13], low and high molecular mass substances [16,17], plasmid and genomic DNA [18] and antibodies [19]. They are also used as the enzyme reactors [20,21] and for the direct synthesis of peptides [22,23]. Most of the separations performed on CIM ion-exchange monolithic columns used a salt gradient, except one separation, which used a pH gradient [24].

The aim of our work was to investigate the applicability and efficiency of CIM disk monolithic columns for the analytical and preparative separation of multiple forms of tomato pectin methylesterase from a tomato fruit extract in order to obtain a sufficient amount of pure enzyme for further experimental work.

2. Experimental

2.1. Chemicals

Tomato pectinesterase (EC 3.1.1.11), MW-SDS-70L (kit for molecular weights 14.000–70.000), Tris(hydroxymethyl)aminomethane (Tris) and Bicinchoninic acid (BCA) kit for protein determination, PhastGel Blue R tablets of Commasie brilliant blue R-350 and 3,5-dinitrosalicylic acid (DNS) were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium hydroxide, sodium chloride, sodium acetate and acetic acid were purchased from Merck (Darmstadt, Germany). Pectin from apples (70–75% degree of esterification) and polygalacturonic acid were obtained from Fluka (Buchs, Switzerland). Phenol red was purchased from Coleman & Bell (Norwood, USA) and potassium sodium tartrate tetrahydrate from Kemika (Zagreb, Croatia).

All of the solutions were prepared with deionized water (Millipore Milli-Q).

The solution of tomato PME from Sigma was prepared as follows: 1 mg/ml of PME in 20 mM Tris buffer (pH 7.4) containing 100 mM NaCl and 750 ng of tomato PME was applied to SDS–PAGE.

2.2. Extraction of PME

Ripe tomato fruits were purchased from the local market and the PME extraction was performed at room temperature. After removal of the peels and the seeds, the tomato flesh (100 g) was homogenized. After the addition of 100 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 0.1 M HCl. Thereafter, the solution was mixed for 5 min by the Ultraturrax. After centrifugation of the solution for 20 min at 12 000 rpm, the pellet was dissolved in 200 ml of water using Ultraturrax. After centrifugation of the solution at 12 000 rpm for 20 min, the pellet was dissolved in 100 ml of water using Ultraturrax and pH was adjusted to 1.6 with diluted hydrochloric acid. After centrifugation of the solution for 20 min at 12 000 rpm, 120 mg of Tris was added to the supernatant and the pH was adjusted to 7.4 by adding 2 M sodium hydroxide. The supernatant obtained after centrifugation for 15 min at 14 000 rpm was filtered through a membrane filter (Millipore Millex–HV, hydrophilic poly(vinylidene difluoride) (PVDF) 0.45 μm) and then injected into an HPLC system or stored at −20 °C.

For comparison, a simple extraction using 100 g of tomato homogenate mixed with 100 ml of 1 M NaCl for 5 min with Ultraturrax followed by centrifugation at 12 000 rpm for 20 min was performed.

The final extraction for preparative purposes was performed according to the first extraction using HCl with 1 kg of tomato fruits.

2.3. Determination of the protein content

The concentration of the proteins in the extract and the HPLC fractions was determined by spectrophotometric Micro BCA Protein Assay (Sigma) using a Bicinchoninic Acid Kit.

2.4. HPLC separation

The HPLC system consisted of a ConstaMetric 4100 pump (Thermo Separation Products (TSP), Riviera Beach, CA, USA), AS3000 autosampler (TSP) with a fixed 100 μl loop for analytical work or a Rheodyne injector (model 7125) with a 3 ml self made loop for semi-preparative work and a SpectroMonitor 3200 UV detector (TSP) set to 280 nm. Separa-

tion was performed on Convective Interaction Media (CIM) disk monolithic columns (diameter, 12 mm; length, 3 mm) bearing sulfonyl (SO_3) or carboxymethyl (CM) cation groups from BIA Separations, Ljubljana, Slovenia. Regeneration of the CIM disk monolithic columns was performed by placing them into a 1 M NaOH water solution.

PME isoenzymes from crude tomato extracts were separated using a linear gradient of sodium chloride on a CIM SO_3 disk monolithic column. The mobile phase consisted of buffer A: 20 mM Tris (2.42 g Tris base per 1 l, neutralised with 10 times diluted concentrated HCl to pH 7.4) and buffer B: buffer A containing 1 M NaCl, pH adjusted to pH 7.4, at a flow rate of 4 ml/min.

The gradient used for the semi-preparative chromatography was as follows: 100% A (1 min), linear gradient from 100% to 30% A (4 min), 30% A to 100% A (0.1 min), 100% A (0.9 min).

Analytical chromatography was performed using the same SO_3 disk and mobile phases as above, but with a different gradient, due to the smaller injection volume of 100 μl: linear gradient from 100% A to 30% A (3 min), 30% A to 100% A (0.1 min), 100% A (0.4 min), at a flow rate of 4 ml/min.

Ultrafiltration (Amicon, 8400) was performed using YM10 (Millipore, Bedford, USA), YM30 and XM50 membranes (DIAFLO ultrafiltration membranes, Amicon, Danvers, MA, USA).

Sodium chloride present in the concentrates XM50 and YM10 and filtrate XM50 (with the isolated PME fraction B, Fig. 1) was removed by passing these solutions through a PD-10 column (prepacked Sephadex G-25 column, Amersham Biosciences, UK). The obtained eluates were filtered through a 0.45 and 0.20 μm membrane filters (Minisart, Sartorius) before the next chromatographic step using two CIM CM disks (in the same housing) using the same mobile phase and the same flow rate used for the separation on the SO_3 disk. For this separation, the following sodium chloride step gradient was used: 100% A (1 min), from 100% A to 90% A (0.1 min), 90% A (3.9 min), 90% A to 100% A (0.1 min), 100% A (1.9 min). Injection volume was 3 ml.

2.5. PME activity assay

Substrate: 0.4 g pectin was dissolved in 80 ml of water while heating. NaCl (1.17 g) was added along with 1 ml of indicator solution (9 mg of phenol red dissolved in 10 ml water by ultrasonication) and adjusted to pH 7.5 (raspberry red colour) with 0.1 M NaOH or a bit of 0.1 M HCl and filled with water up to a total volume of 100 ml. For the visual estimation of the activity, 50 μl of the test solution was mixed with 0.5 ml of the substrate and a colour change from red to yellow indicated activity.

2.6. PG activity assay

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

2.7. Polyacrylamide gel electrophoresis (SDS–PAGE)

The purity and molecular masses of PME were determined by SDS–PAGE on a slab gel prepared with 15% (resolving gel) and 4% (stacking gel) acrylamide by the Laemmli method [25]. The samples of crude tomato extract, isolated protein fractions and tomato PME from Sigma were applied in parallel with a protein standard marker MW-SDS-70L (kit for molecular masses, 14 000–70 000). After electrophoresis, the gels were subjected to silver staining according to the procedure of Heukeshoven and Dernick [26].

3. Results and discussion

Two extractions of the PME isoenzymes from 100 g of fresh tomato fruits were compared in order to choose the optimal extraction conditions. The first extraction was performed according to Pressey and Woods [6] with a minor modification in the last step. During this step, the addition of NaCl followed by ultrafiltration were omitted. Instead, the extract was prepared for chromatography by adding Tris buffer (20 mM in the final extract) and adjusting the pH to 7.4 by adding NaOH. An additional difference was that the extraction was performed at room temperature and not at 4 °C.

Table 1
PME activity of chromatographic fractions obtained after fractionation of two differently prepared extracts of fresh tomato on a CIM SO_3 disk monolithic column (Fig. 1, linear gradient)

Fraction no.	HCl extract	NaCl extract
1	–	–
2	±	+
3	±	±
4	±	++
5	–	+
6	–	+
7	–	+
8	–	–
9	–	–
10	–	–
11	–	–
12	–	–
13	–	–
14	+	+
15	+++	+++
16	+++	+++
17	+	+
18	+	–
19	±	++
20	±	+
21	±	–
22	–	–
23	–	–
24	–	–
25	–	–

Activity was determined using the substrate with 200 mM NaCl.

The second extraction was a simple extraction with 1 M NaCl. Both extracts ("HCl extract" and "NaCl extract") were diluted ten times after centrifugation and the chromatographic step was performed as described for the semi-preparative HPLC. Fractions were collected in 10 s intervals with time 0 being the beginning of the chromatographic run. PME activity of the collected fractions was estimated visually (Table 1) with the substrate prepared without NaCl and with 200 mM NaCl [6,9].

Fractions of the "NaCl extract" showed stronger PME activity than the fractions of the "HCl extract". This is particularly the case for the fractions at the beginning and near the end of the fractionation (Table 1). Additionally, only fractions 15 and 16 (fraction B, Fig. 1) showed PME activity when analysed with the substrate prepared without NaCl, which proved that only these fractions contained "salt independent PME isoenzyme" [6,9]. This experiment also proved that PME from peak A (Fig. 1) is not the same as in peak B (Fig. 1) and does not originate from disk overloading. Fraction 19 (fraction C, Fig. 1) had PME and PG activity. The "HCl extract" contained fewer proteins than the "NaCl extract", while the content of the salt independent PME isoenzyme, which was chosen for the isolation, was similar in both extracts. However, due to the low content of NaCl, "HCl extract" was directly applicable for the semi-preparative cation-exchange chromatography on CIM SO_3 disk monolithic columns, which was not the case for the "NaCl extract". For these reasons, the final extraction of PME isoenzymes from 1 kg of fresh tomato was performed using the extraction method with HCl.

The obtained crude extract from 1 kg of fresh tomato fruit was fractioned by the HPLC system via the 3 ml loop in conditions described for the semi-preparative HPLC. The targeted enzymes were separated from the remaining proteins by means of cation-exchange chromatography using CIM SO_3 disk monolithic columns, Tris buffer (20 mM, pH 7.4) and a

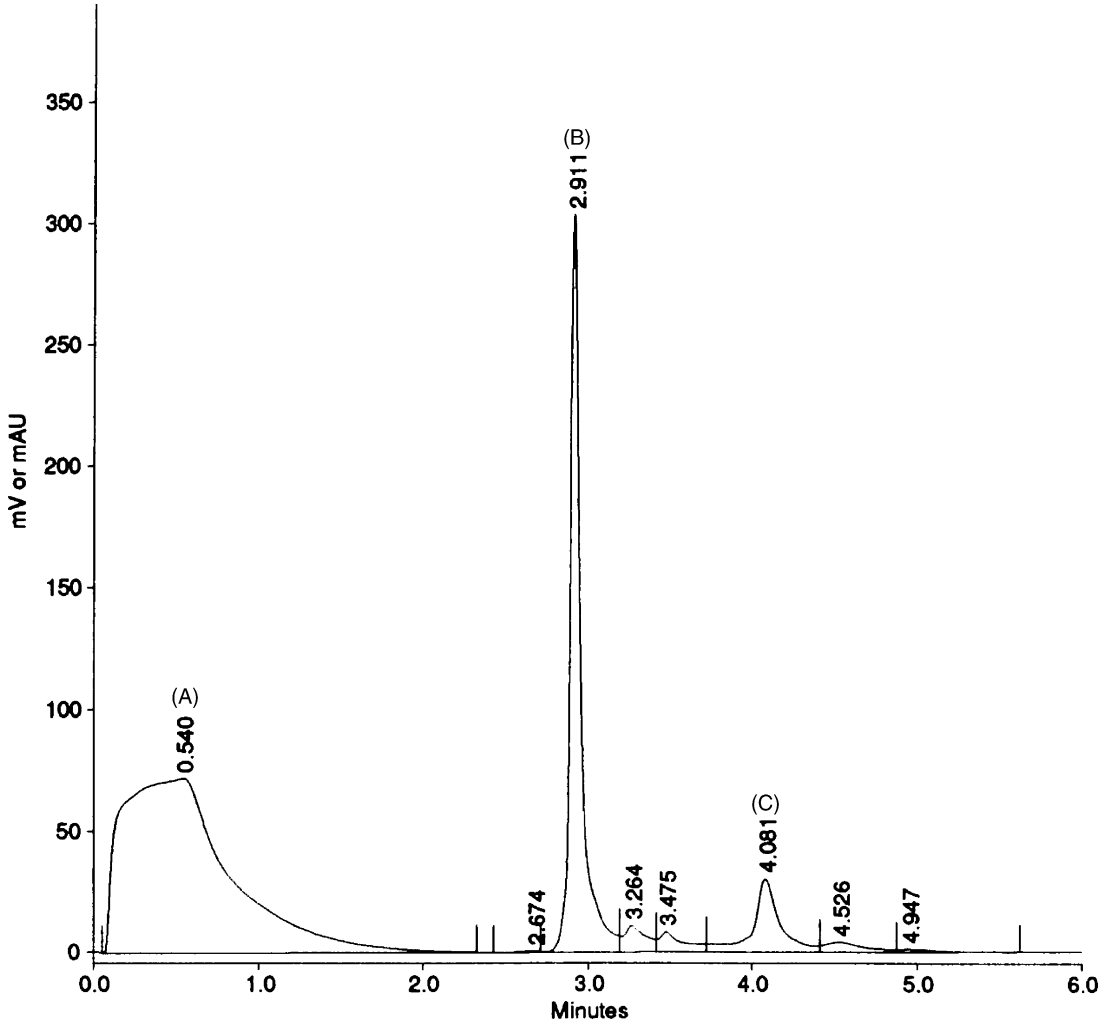

Fig. 1. Semi-preparative HPLC separation of PME isoenzymes from crude tomato extract on a CIM SO_3 disk monolithic column (diameter, 12 mm; length, 3 mm) using a linear gradient elution and injection volume of 3 ml. All three peaks A, B (salt independent PME activity) and C showed PME activity. PG activity was detected in peak C. 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: 100% A (1 min), 0–70% B linear in 4 min, 30–100% A in 0.1 min, 100% A (0.9 min).

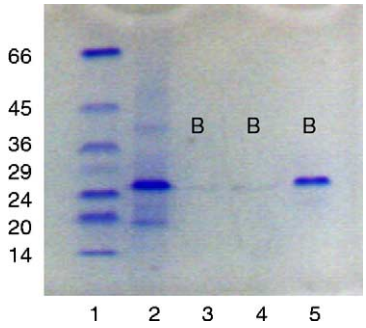

Fig. 2. SDS–PAGE (15%) with coomassie blue staining for purity control of PME isolated from the crude extract. Lanes: (1) standard protein marker (values in $M_r \times 10^3$ at left-hand side); (2) tomato crude extract; (3–5) different applications of fraction B (Fig. 1).

linear sodium chloride gradient. It was demonstrated that the separation is insensitive to slight differences in the pH value of the mobile phase. Fig. 1 shows the obtained chromatogram for the separation of proteins from the crude tomato extract. The fractions indicated by all three chromatographic peaks (A–C) showing PME activity were collected. The concentration of proteins was determined in the extract and in the HPLC fractions A–C. It was proven that it is essential to mix (vortex) the sample solutions just before taking an aliquot for the assay.

To check the purity of the isolated fractions, the crude extract, the isolated fractions A–C, and the Sigma PME tomato standard were applied to the SDS–PAGE with coomassie blue staining (Fig. 2). The molecular mass of the PME isoenzyme in fraction B was estimated to be 26 000 (above the standard marker of 24 000), which is in any case more than 24 000 as estimated by Pressey and Woods [6]. The results from the SDS–PAGE using more sensitive silver staining showed several bands for fractions A and C (Fig. 3). In addition to PME activity, fraction C also showed PG activity, which is according to the literature [27], probably related to the band

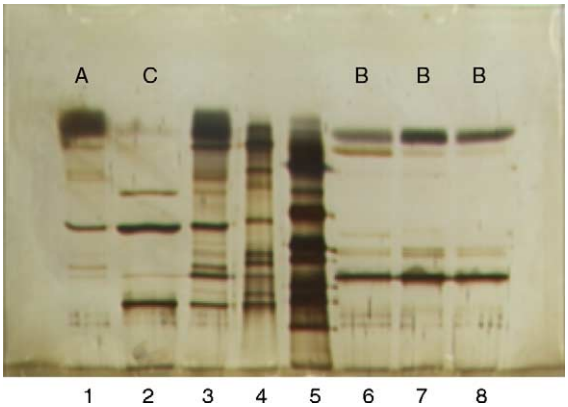

Fig. 3. SDS–PAGE (15%) with silver staining for purity control of PME fractions isolated from the crude extract on a CIM SO_3 disk monolithic column (Fig. 1, linear gradient). Lanes: (1) fraction A; (2) fraction C; (3) tomato crude extract; (4) tomato PME from Sigma; (5) standard protein marker; (6–9) combined fractions B from 10 chromatographic runs.

at approximately 43 kDa. Because of the additional bands (at the top of the gel) in lanes 6–8 (Fig. 3) for combined fractions B from 10 chromatographic runs, an additional purification step was necessary to obtain pure targeted PME from fraction B which contained the largest amount of PME (Fig. 1).

Because of the sharp elution from the CIM disk (Fig. 1, elution volume less than 1 ml against injection volume of 3 ml!) all the fraction B's with already concentrated PME were combined and concentrated by means of ultrafiltration by first using a cut-off YM30 membrane. The obtained YM30 concentrate and YM30 filtrate were analysed by analytical HPLC. About 90% of the total PME mass obtained after ultrafiltration remained in the YM30 concentrate. The YM30 filtrate was further subjected to ultrafiltration using a cut-off YM10. In order to separate the upper bands (Fig. 3, lanes 6–8) from the targeted PME, another ultrafiltration step of

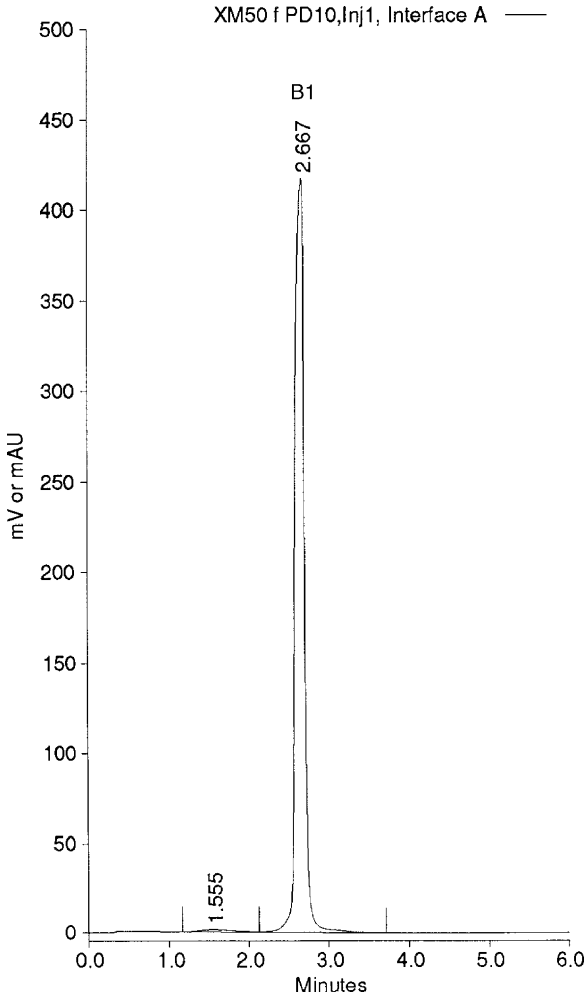

Fig. 4. Purification of PME isoenzyme from fraction B (Fig. 1) on a CIM CM column (two disks with diameter, 12 mm and length, 3 mm) using step gradient elution and injection volume, 3 ml. Peak B1 showed salt independent PME activity. Binding buffer (A): 20 mM Tris, pH 7.4; elution buffer (B): buffer A containing 1 M NaCl, pH 7.4; flow rate: 4 ml/min; gradient: 100% A (1 min), 0–10% B in 0.1 min, 10% B (3.9 min), 90–100% A in 0.1 min, 100% A (1.9 min).

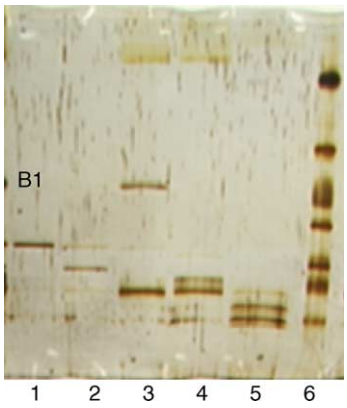

Fig. 5. SDS–PAGE (15%) with silver staining for purity control of the targeted PME, fraction B1 (Fig. 4) purified from fraction B (Fig. 1) on a CIM CM disk monolithic column (Fig. 4, step gradient). Lanes: (1) targeted PME, fraction B1 and (6) standard protein marker.

the YM30 concentrate using a cut-off XM50 was performed which resulted in about 16% proteins (estimated by protein analysis) in the XM50 concentrate. The obtained 2.5 ml of concentrate XM50 and 22 ml of filtrate XM50 with the protein content being 450 and 280 μg/ml, respectively, showed PME activity and also gave a PME peak when analysed by HPLC. The YM10 filtrate also showed a minor peak at the same retention time as PME, but did not have PME activity and was only an impurity with a low molecular mass. Purity testing of the XM50 and YM10 concentrates and XM50 filtrate by silver staining SDS–PAGE showed that an additional purification step is needed and that the ultrafiltration through YM30 and XM50 should be omitted potentially speeding up the whole procedure and increasing the final yield.

The obtained solutions of the XM50 concentrate and filtrate, and YM10 concentrate were stored at −20 °C. Before the final chromatographic purification these solutions were desalted via a PD10 column and filtered through 0.45 and 0.20 μm membrane filters. The targeted PME was further purified by an additional chromatographic step by using a weaker cation-exchanger CIM–carboxymethyl (CM) disk monolithic column and a step gradient. Due to the relatively high sample loading, two disks were inserted into the housing to increase the capacity of the column. As can be seen in Fig. 4, 10% of mobile phase B (100 mM NaCl) was enough to elute the targeted enzyme from the disks. Purity testing of the isolated targeted PME enzyme (B1, Fig. 4) by silver staining SDS–PAGE (lane 1, Fig. 5) showed that the last purification step was successful. The yield was 7.5 mg of purified salt in-

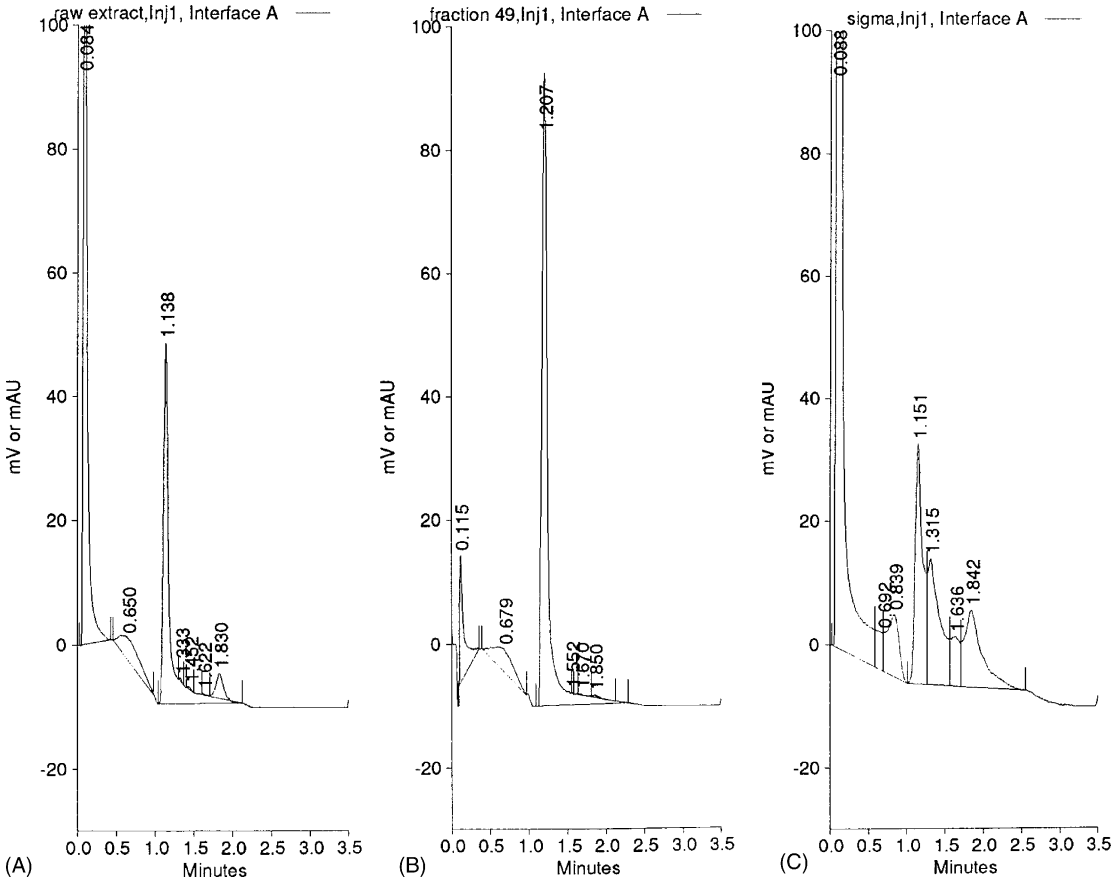

Fig. 6. Analytical HPLC separation of PME isoenzymes on a CIM SO_3 disk monolithic column using a linear gradient elution and injection volume of 100 μl. Samples: tomato crude extract (A) fraction B1 from Fig. 4(B) and tomato PME from Sigma (C). Binding buffer (A): 20 mM Tris, pH 7.4, elution buffer (B): buffer A containing 1 M NaCl, pH 7.4; flow rate: 4 ml/min; gradient: 0–70% B linear in 3 min, 30–100% A in 0.1 min, 100% A (0.4 min).

dependent PME isoenzyme isolated from 1 kg of fresh tomato fruits.

The isolated PME isoenzyme corresponded to the PME in fractions 15 and 16 from the preliminary experiments (Table 1). The salt independent activity (at pH 7.4) of the isolated PME is similar to those reported for isoenzymes (PEIV: M_r 24 000 [6]; PME1: 31 000 [7]; PE-A: 36 000 [8]; PME1a and PME1b: both 34 000 [9]), but with different molecular masses which were all estimated from SDS–PAGE. SDS–PAGE of tomato PME standard from Sigma (Fig. 3, lane 4) previously investigated by Savary [9] showed many components including those appearing also in our extracts. This was additionally confirmed by an analytical HPLC separation of PME isoenzymes from tomato crude extract, fraction B1 and tomato PME from Sigma (Fig. 6). The observed differences are explained by the fact that different PME forms and different contentration of these forms depend on the tomato variety and other conditions (ripeness degree, soil and climate conditions, etc.). Extraction conditions can influence the yield of the individual forms, especially when harsh conditions as in our case (low pH!) are applied. It might not be a coincidence that the extraction according to Pressey and Woods [6] resulted in the PME with the lowest molecular mass.

During the chromatographic work, HPLC peak distortions appeared from time to time. The problem was solved by a simple regeneration of CIM SO_3 and CM disk monolithic columns by removing them from the housing, putting them in a 1 M NaOH water solution and leaving them for at least 1 h at room temperature. Refrigerating the disks for several days in the same solution did not have any deleterious effect on the disks. Before the chromatographic separation, the regenerated disk was first washed with water, inserted into the housing, attached to the HPLC system, and washed with mobile phase for at least one run.

Ion-exchange chromatography is common to all published procedures for the isolation of PME isoforms from tomato fruit extracts, e.g. ion-exchange on DEAE–cellulose followed by affinity chromatography on Heparin Sepharose [7], four chromatographic steps: 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 [6]. Compared to the published procedures for the isolation and purification of PME from plant materials, our new procedure using CIM disk monolithic columns provides a much faster and more efficient way to perform this procedure. Nevertheless, further investigation is needed to isolate and purify the PME forms from fractions A and C (Fig. 1). However, due to the rather time consuming chromatography of large amounts of tomato extracts on small disks, the semi-preparative chromatographic method for the separation of PME isoenzymes should be scaled-up to the CIM tube monolithic columns. This can be done using the BIA Separations method transfer calculator, which is based upon a formula, derived from the transfer of gradient methods for protein separation between columns of different size [28].

Furthermore, our work showed a potential application of short monolithic columns in analysis and semi-preparative work. Additionally, the high separation speed enables fast method development and the separation procedures for the CIM disks serve as a basis for scaling up the separation procedures to be used for the separation of proteins from larger extract amounts.

Acknowledgement

This study has been carried out with the financial support from the Ministry of Education, Science and Sport of the Republic of Slovenia (grant L1-6600-0104) and from the Commission of the European Union, specific RTD programme “Quality of Life and Management of Living Resources”, contract no. QLK1-CT-2002-71361, “Development of new food additives extracted from the solid residue of the tomato processing industry for the application in functional food”.

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