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Enzyme-aided extraction of lycopene from high-pigment tomato cultivars by supercritical carbon dioxide

Marcello Salvatore Lenucci [a,*], Monica De Caroli [a], Pier Paolo Marrese [a], Andrea Iurlaro [a], Leonardo Rescio [b], Volker Böhm [c], Giuseppe Dalessandro [a], Gabriella Piro [a]

[a] Dipartimento di Scienze e Tecnologie Biologiche ed Ambientali (Di.S.Te.B.A.), Università del Salento, via Prov.le Lecce-Monteroni, 73100 Lecce, Italy
[b] PIERRE s.r.l., 73013 Galatina (LE), Italy
[c] Institute of Nutrition, Friedrich Schiller University Jena, Dornburger Straße 25-29, 07743 Jena, Germany

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ABSTRACT

This work reports a novel enzyme-assisted process for lycopene concentration into a freeze-dried tomato matrix and describes the results of laboratory scale lycopene supercritical CO_2 (SC-CO_2) extractions carried out with untreated (control) and enzyme-digested matrices. The combined use of food-grade commercial plant cell-wall glycosidases (Celluclast/Novozyme plus Viscozyme) allows to increase lycopene (~153%) and lipid (~137%) concentration in the matrix and rises substrate load onto the extraction vessel (~46%) compared to the control. The addition of an oleaginous co-matrix (hazelnut seeds) to the tomato matrix (1:1 by weight) increases CO_2 diffusion through the highly dense enzyme-treated matrix bed and provides lipids that are co-extracted increasing lycopene yield. Under the same operative conditions (50 MPa, 86 °C, 4 mL min^{-1} SC-CO_2 flow) extraction yield from control and Celluclast/Novozyme + Viscozyme-treated tomato matrix/co-matrix mixtures was similar, exceeding 75% after 4.5 h of extraction. However, the total extracted lycopene was ~3 times higher in enzyme-treated matrix than control.

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1. Introduction

Lycopene, the red pigment synthesised and stored in tomato berry chromoplasts, is one of the over 750 carotenoids found in nature (Britton, 2004). It is used in food, pharmaceutical and cosmetic industry as natural dye, antioxidant, cancer-preventing and anti-ageing product. A number of *in vitro*, *in vivo* and *ex vivo* studies have demonstrated that lycopene supplementation is inversely associated to cardiovascular diseases, cancer risk and diabetes, through its powerful antioxidant activity alongside other, not yet fully understood, mechanisms of action (Kong et al., 2010; Palozza, Simone, Catalano, Russo, & Böhm, 2012). Lycopene extraction with supercritical carbon dioxide (SC-CO_2) from red-ripe tomato berries is an excellent technique suitable to replace the use of harmful organic solvents and satisfy the increasing demand for biological solvent-free lycopene (Konar, Haspolat, Poyrazoğlu, Demir, & Artık, 2012). The preparation of a freeze-dried tomato matrix is a key step to maximize the efficiency and yield of lycopene extraction by SC-CO_2 (Lenucci et al., 2010). In the tomato matrix, consisting of dehydrated clumps of berry mesocarp parenchyma cells, lycopene is present as red crystals within the chromoplasts. Lycopene crystals are enclosed into newly synthesised membranes originating by introflections of the inner membrane plastid envelope (Simkin, Zhu, Kuntz, & Sandmann, 2007). Thus, to solubilise and extract lycopene, the supercritical fluid must reach these organelles penetrating through the pecto-cellulosic primary cell-wall and the membrane bilayers of cell which represent potential obstacles to fluid free diffusion.

Enzymes have been extensively used to improve the yield and quality of several plant natural products, including flavouring, colorants and bioactives [see Sowbhagya and Chitra (2010) and Puri, Sharma, and Barrow (2012) for excellent reviews]. Pre-treatments with cell wall hydrolases have proved effective in enhancing the extraction rate and quality of oily products from a variety of oleaginous seeds and fruits by mechanical or organic solvent based methods (Domínguez, Núñez, & Lema, 1994). Since the primary cell wall of dicotyledonous plants comprises mainly of cellulose, hemicelluloses and pectins, most of the works in this field utilise cocktails of cellulase, xyloglucanase and pectinase to hydrolyse

* Corresponding author. Tel.: +39 0832 298612; fax: +39 0832 298858.
E-mail addresses: marcello.lenucci@unisalento.it (M.S. Lenucci), monica.decaroli@unisalento.it (M. De Caroli), pierpaolo.marrese@unisalento.it (P.P. Marrese), iurlaro.andrea@libero.it (A. Iurlaro), pierrechimica@libero.it (L. Rescio), Volker.Boehm@uni-jena.de (V. Böhm), giuseppe.dalessandro@unisalento.it (G. Dalessandro), gabriella.piro@unisalento.it (G. Piro).

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and degrade the polysaccharide network surrounding the cell, improving, in this way, the release of intracellular contents by hydro-distillation or conventional solvent extraction (CSE). Following this approach, enzymatic mixtures have been explored as a mean to enhance the extraction of capsaicinoids and carotenoids from chilli pepper (Santamaría et al., 2000), α- and β-carotene from orange peel, sweet potatoes and carrots (Çinar, 2005), lycopene from tomato tissues (Choudhari & Ananthanarayan, 2007; Zuorro, Fidaleo, Lavecchia, 2011; Papaioannou & Karabelas, 2012) lutein from marigold flowers (Barzana et al., 2002), and flavonoids from *Ginkgo biloba* (Chen, Xing, Huang, & Xu, 2011) or pigeonpea (Fu et al., 2008) leaves. However, as far as we know, the combined use of cell-wall hydrolytic enzymes and supercritical fluids to extract plant metabolites has been only marginally explored. Recently, a similar approach has been assessed for the enhancement of supercritical fluid extraction of grape seed oil obtaining a considerable increase (+44%) in oil yield compared to untreated seeds (Passos, Silva, Da Silva, Coimbra, & Silva, 2009).

The present work reports a novel enzyme assisted process for the deconstruction of primary cell-wall of tomato parenchyma cells aimed at the preparation of a matrix suitable for an improved lycopene extraction by SC-CO_2. In addition, we describe the results of laboratory scale SC-CO_2 extraction tests carried out with control and treated tomato matrices, pure or blended with different co-matrices, in order to optimise lycopene extraction yield.

This work is the first comprehensive report on the possible use of hydrolytic enzyme pretreatments for SC-CO_2 extraction of carotenoids from plants. It also suggests some technological solutions to overcome the problems related to the excessive packing density of the enzyme digested tomato matrices.

2. Materials and methods

2.1. Chemicals

Celluclast 1.5 L (Cellulase from *Trichoderma reesei*, declared activity 700 EGU g^{-1}), Novozyme 188 (Cellobiase from *Aspergillus niger*, declared activity ⩾250 U g^{-1}), Viscozyme L (multi-enzyme complex containing a wide range of carbohydrases, including arabanase, cellulase, β-glucanase, hemicellulase, xylanase and pectinase from *Aspergillus* sp., declared activity ⩾100 FBG g^{-1}) and Flavourzyme 500 L (Protease from *Aspergillus oryzae*, declared activity ⩾500 U g^{-1}) were from Novozymes A/S (Bagsvaerd, Denmark).

(all-*E*)-lycopene standard was purchased from CaroteNature (Ostermundigen, Switzerland), the other lycopene (*Z*)-isomers were prepared by using iodine or heat isomerisation and fractionation using preparative C_{30}-HPLC as described by Fröhlich, Conrad, Schmid, Breithaupt, and Böhm (2007), and showed a purity of 97–99%. All the HPLC grade solvents were purchased from Sigma–Aldrich (Milan, Italy). High purity carbon dioxide (99.995%) for supercritical fluid extraction was purchased from Mocavero Ossigeno (Lecce, Italy).

2.2. Plant material

All experiments were carried out using a tomato purée prepared from red-ripe tomatoes of the high-pigment cultivar HLY 18 (COIS '94 Srl, Belpasso, Italy) as previously described (Lenucci et al., 2010). This variety, obtained by conventional breeding techniques, is characterised by a high lycopene content.

Briefly, tomatoes were subjected to extensive washing under running water, blanched in water at 70 °C for 5 min, crushed and sieved by a Reber 9004 N tomato squeezer (Reber, Luzzara, Italy) in order to obtain a tomato purée made up of cell clumps of similar size to the sieve used (1 mm), separated from skins, seeds and vascular tissues. Subsequently, the tomato purée was packed into 1 L screw top glass jar, pasteurised at 121 °C, and stored in the dark at room temperature until use.

2.3. Tomato purée digestion and matrix preparation

For studies aimed to assess the effect of enzyme concentration and digestion time on freeze-dried matrix weight and lycopene titer, experiments were carried out in small volumes. Triplicate aliquots (50 mL) of tomato purée were digested with 0–2% v/v of: Celluclast/Novozyme (3:1 by vol.), Viscozyme or Flavourzyme, for 0–48 h, at 50 °C, under constant stirring. Enzyme-treated tomato purées were centrifuged at 27,000*g* for 10 min by using a J2-21 Beckmann centrifuge (Beckman Coulter, Fullerton, CA, US) to remove water soluble substances. The pellets were dehydrated to constant weight by a Christ ALPHA 2-4 LSC freeze-dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). Freeze-dried tomato matrices were ground in a laboratory ultra centrifugal mill (ZM200, Retsch GmbH, Haan, Germany) through 35 mesh (500 μm) sieve, vacuum-packaged in food grade oxygen impermeable plastic bags and stored in a freezer at −20 °C. The procedure was scaled-up to obtain an amount of lyophilised tomato matrix sufficient to perform pilot-scale SC-CO_2 extractions of lycopene. In this case the freeze-dried matrices were prepared by digesting, for 24 h, at 50 °C, under constant stirring, 1L of tomato purée with 0.25% v/v of: (1) Celluclast/Novozyme (3:1 by vol.); (2) Viscozyme; (3) Flavourzyme; (4) Celluclast/Novozyme (3:1 by vol.) + Viscozyme; (5) Celluclast/Novozyme (3:1 by vol.) + Flavourzyme; (6) Celluclast/Novozyme + Viscozyme + Flavourzyme. A control matrix was prepared from undigested tomato purée.

2.4. Residual moisture determination

Residual moisture in freeze-dried tomato matrices was measured gravimetrically, on 1.0 g aliquots (three independent replicates), after further drying at 105 °C to constant weight in a Büchi TO-50 infrared drier (Büchi Labortechnik AG, Postfach, Switzerland).

2.5. Determination of lycopene, total lipids and total proteins in the freeze-dried matrices

Triplicate aliquots of each freeze-dried tomato matrix (50 mg) were used for the extraction of lycopene by the method of Perkins-Veazie, Collins, Pair, and Roberts (2001). All extractions were carried out in the dark to prevent lycopene degradation and/or isomerisation.

Total lycopene content was determined by HPLC as previously described (Tlili et al., 2011). Briefly, carotenoids were extracted with 0.05% (w/v) BHT in acetone and 95% ethanol (1:1, v/v), separated by partition into hexane and directly assayed by a Dionex HPLC (Dionex s.r.l., Milan, Italy) with an AD 25 UV–Vis detector. The separation was performed at 31 °C on an Acclaim HPLC column C_{18} (5 μm, 250 × 4.6 mm) by using a linear gradient of acetonitrile (A), hexane (B) and methanol (C) as follow: from 70% A, 7% B, 23% C to 70% A, 4% B, 26% C within 35 min, with a flow rate of 1.5 mL min^{-1}. Peaks were detected at 503 nm.

Lycopene isomers were analysed, in the same above mentioned extracts, using a Merck-Hitachi (Darmstadt, Germany) HPLC system (pump L-7100, degasser L-7614, autosampler L-7200, diode array detector L-7450, interface L-7000) and a Jetstream plus column oven (JASCO, Gross-Umstadt, Germany). An analytical polymeric C_{30}-column [YMC Carotenoid S, 5 μm, 250 × 4.6 mm (YMC Europe, Dinslaken, Germany)], preceded by a C_{18} ProntoSil

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