Food Chemistry 139 (2013) 631–639

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Biosynthesis of highly enriched ^{13}C-lycopene for human metabolic studies using repeated batch tomato cell culturing with ^{13}C-glucose

Nancy Engelmann Moran [a], Randy B. Rogers [b], Chi-Hua Lu [e,1], Lauren E. Conlon [c], Mary Ann Lila [b,d], Steven K. Clinton [a], John W. Erdman Jr. [c,e,*]

[a] Division of Medical Oncology, Department of Internal Medicine, and the Comprehensive Cancer Center, The Ohio State University, 320 W 10th Avenue, Columbus, OH 43210, USA
[b] Department of Natural Resources and Environmental Sciences, The University of Illinois, 1201 S. Dorner Dr., Urbana, IL 61801, USA
[c] The Division of Nutritional Sciences, The University of Illinois, 905 S. Goodwin Ave., Urbana, IL 61801, USA
[d] Plants for Human Health Institute, North Carolina State University, 600 Laureate Way, Kannapolis, NC 28081, USA
[e] Department of Food Science and Human Nutrition, 905 S. Goodwin Ave., The University of Illinois, Urbana, IL 61801, USA

ARTICLE INFO

Article history:
Received 30 July 2012
Received in revised form 21 November 2012
Accepted 10 January 2013
Available online 23 January 2013

Keywords:
Carotenoids
Tracers
Lycopene
Tomato
Nutrition
Plant cell culture

ABSTRACT

While putative disease-preventing lycopene metabolites are found in both tomato (*Solanum lycopersicum*) products and in their consumers, mammalian lycopene metabolism is poorly understood. Advances in tomato cell culturing techniques offer an economical tool for generation of highly-enriched ^{13}C-lycopene for human bioavailability and metabolism studies. To enhance the ^{13}C-enrichment and yields of labelled lycopene from the *hp-1* tomato cell line, cultures were first grown in ^{13}C-glucose media for three serial batches and produced increasing proportions of uniformly labelled lycopene (14.3 ± 1.2%, 39.6 ± 0.5%, and 48.9 ± 1.5%) with consistent yields (from 5.8 to 9 mg/L). An optimised 9-day-long ^{13}C-loading and 18-day-long labelling strategy developed based on glucose utilisation and lycopene yields, yielded ^{13}C-lycopene with 93% ^{13}C isotopic purity, and 55% of isotopomers were uniformly labelled. Furthermore, an optimised acetone and hexane extraction led to a fourfold increase in lycopene recovery from cultures compared to a standard extraction.

1. Introduction

Epidemiological and laboratory studies suggest that tomato (*Solanum lycopersicum*) consumption may reduce the risk for a number of chronic diseases including cardiovascular disease and cancers, notably prostate cancer (Giovannucci et al., 1995; Rissanen, Voutilainen, Nyyssonen, & Salonen, 2002; WCRF & AICR, 2007). Tomatoes are a dietary source of a variety of phytochemicals including the red carotenoid lycopene, a linear tetraterpene. Lycopene along with its carotenoid precursors, phytoene and phytofluene, derived from tomatoes, have numerous hypothesised bioactivities that may mediate health promotion, including participation in antioxidant defence systems (Canene-Adams, Campbell, Zaripheh, Jeffery, & Erdman, 2005; Engelmann, Clinton, & Erdman, 2011). Moreover, an array of lycopene metabolites have been identified in human plasma and tissues (Khachik et al., 2002; Kopec et al., 2010), but the origin of these potentially bioactive metabolites is unknown. Plants have many carotenoid cleavage dioxygenase (CCD) enzymes responsible for carotenoid metabolism (Auldridge, McCarty, & Klee, 2006), while in humans only two cleavage enzymes are known to impact carotenoid metabolism. B-carotene monooxygenase 1 (BCMO-1) is responsible for central cleavage of some pro-vitamin A carotenoids, and carotenoid dioxygenase 2 (BCDO-2) primarily acts to eccentrically cleave carotenoids (von Lintig, 2012). The role of these mammalian enzymes in the metabolism of lycopene is only beginning to be elucidated (Ford, Clinton, von Lintig, Wyss, & Erdman, 2010). Therefore, lycopene metabolism occurs in both plant and animal tissues, yet it is currently unknown whether the series of chain-shortened lycopene metabolites previously detected in human plasma are endogenously produced following consumption of lycopene from tomato products and/or if the pre-formed metabolites are absorbed directly from tomato-containing foods (Kopec et al., 2010). A source of inexpensive, isotopically-labelled lycopene will greatly enhance

Abbreviations: APCI, atmospheric pressure chemical ionisation; CP, carotenoid production; CPTA, 2-(4-chlorophenyl-thio)triethylamine; HPLC–PDA, high pressure liquid chromatography–photodiode array; HPLC-RID, high-pressure liquid chromatography refractive index detector; LC–MS, tandem liquid chromatography and mass spectrometer.

* Corresponding author. Address: 455 Bevier Hall, 905 S. Goodwin Ave., Urbana, IL 61801, USA. Tel.: +1 217 333 2527; fax: +1 217 333 9368.

E-mail addresses: nancy.moran@osumc.edu (N.E. Moran), rbrogers@illinois.edu (R.B. Rogers), luchi@missouri.edu (C.-H. Lu), lconlon2@illinois.edu (L.E. Conlon), maryann_lila@ncsu.edu (M.A. Lila), steven.clinton@osumc.edu (S.K. Clinton), jwerdman@illinois.edu (J.W. Erdman Jr.).

[1] Currently at the Division of Animal Sciences, University of Missouri, Columbia, MO 65211, USA.

0308-8146/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodchem.2013.01.016

our ability to better discriminate between endogenous pools of lycopene and newly ingested molecules, while also allowing us to identify metabolites produced in humans (Yeum & Russell, 2002).

Stable and radioactive hydrogen and carbon isotopes have been successfully incorporated into carotenoids for clinical tracer investigations of carotenoid absorption and metabolism (Kelm et al., 2001; Novotny, Kurilich, Britz, & Clevidence, 2005; Tang, Qin, Dolnikowski, Russell, & Grusak, 2005, 2009; Tang et al., 2005). Carotenoids can be either extrinsically labelled by chemical incorporation of isotopes, or intrinsically labelled by the biological incorporation of isotopes, resulting in the labelled carotenoid housed within a labelled plant–food matrix (van Lieshout, West, & van Breemen, 2003). A hybrid of the two methods is achieved when a biolabelled carotenoid is isolated from a biological matrix and utilised as an extrinsic dose. Intrinsically-labelled plant material allows for investigations related to bioavailability of a carotenoid from within the plant food matrix, but because plant-produced labelled carotenoid metabolites are also already present in that tissue, one cannot use the tracer to characterise the production of or to identify human carotenoid metabolic products. Purified, extrinsic dosing experiments can provide insight into the metabolism of a tracer compound over time, allowing for the identification of novel metabolites.

Currently very few, if any, chemically-synthesized, extrinsically labelled carotenoids are commercially-available, so the majority of recent clinical trials have used intrinsically labelled plant material (Kelm et al., 2001; Novotny et al., 2005; Tang et al., 2005; Tang et al., 2005, 2009). Advances in D_2O-hydroponic and $^{13}CO_2$-growth chamber methodologies have generated intrinsically labelled kale, miniature tomatoes, golden rice, and maize for absorption and metabolism studies of lutein, beta-carotene, and lycopene. These tools have advanced our understanding of carotenoid bioavailability and human conversion of pro-vitamin A carotenoids to vitamin A, but relatively low isotopic enrichment (Tang et al., 2005, 2009) and long exposure times necessary to intrinsically label plant reproductive tissues (i.e. tomato) (van Lieshout et al., 2003) make these impractical for production of highly enriched lycopene tracers for the discovery and pharmacokinetic characterisation of low-concentration lycopene metabolites.

Plant cell culture offers an option for producing isotopically labelled plant secondary metabolites for nutritional studies. Plant cell culture lends itself to labelled molecule production by allowing for intense environmental control for optimal metabolite production, as well as labelled precursor feeding for isotope incorporation (Lila, 2004). *In vitro* ^{14}C and ^{13}C labelling strategies have been applied for production of labelled isoflavones, anthocyanins, and stilbenes (Engelmann, Reppert, Yousef, Rogers, & Lila, 2009; Grusak, Rogers, Yousef, Erdman, & Lila, 2004; Krisa et al., 1999; Reppert, Yousef, Rogers, & Lila, 2008; Vitrac et al., 2002; Yousef et al., 2004), with ^{13}C-resveratrol production from grape cultures reaching semi-commercial yields (Yue, Zhang, & Deng, 2011).

A number of recent advances have been made in the area of isotopically-labelled tomato carotenoid production from tomato cell cultures, with utilisation of different herbicide treatment protocols for desired carotenoid accumulation profiles (Campbell, Rogers, Lila, & Erdman, 2006; Engelmann, Rogers, Lila, & Erdman, 2009; Engelmann et al., 2010). The identification of a high-lycopene yielding tomato cell line for ^{13}C-lycopene production has significant potential for producing highly enriched ^{13}C-lycopene due to the ability to grow a small amount of unlabeled inoculum in a carotenoid production (CP) medium containing 100% uniformly labelled ^{13}C-glucose as the carbon source (Engelmann et al., 2010). In a previous report, we observed a distribution of ^{13}C-lycopene isotopomers with a yield of ~3.5 mg ^{13}C-lycopene/L culture, with the uniformly labelled isotopomer being most prominent of all isotopomers (Engelmann et al., 2010). These cultures were single-batch cultured such that cells were grown for one CP cycle with labelled glucose and then harvested. However, for human clinical investigations of lycopene absorption and metabolism using ^{13}C tracers from plant cell culture to be economically and temporally feasible, greater yields of more highly enriched material is needed.

In the current study we demonstrate that the previously-described *in vitro hp-1* tomato cell lycopene production system (Engelmann et al., 2010) can be repeatedly batch cultured for up to three growth cycles in CP media containing the lycopene cyclase inhibitor, CPTA, for consistent lycopene yields. This serial culturing strategy was then utilised for ^{13}C-lycopene labelling, using uniformly labelled ^{13}C-glucose as the labelled precursor material. This approach resulted in substantially increased proportions of uniformly labelled lycopene ($^{13}C_{40}H_{56}$) with each repeated culturing cycle, as confirmed by LC–MS. Based on the observation that using cells previously grown in labelled media as inoculum for a subsequent labelling phase significantly increases $^{13}C_{40}H_{56}$ isotopomeric purity, we conducted a two phase time-course study, identifying the optimal duration for a ^{13}C-loading phase for generation of a maximal amount of ^{13}C-labelled inoculum, and the optimal duration of a ^{13}C-lycopene labelling phase, based on lycopene yield and labelled glucose utilisation. The most favourable conditions were then tested in a two phase loading and labelling trial, resulting in ^{13}C-lycopene consisting of 55% uniformly labelled lycopene and a mixture of other highly enriched isotopomers. Further tracer production efficiency was achieved by developing and testing a more thorough lycopene extraction protocol. In this study, we demonstrate novel approaches for the efficient production of highly enriched ^{13}C-lycopene.

2. Materials and methods

2.1. Plant material and cell culturing methods

Tomato (*Solanum lycopersicum*) cell suspension cultures were previously established from the high lycopene mutant 'Ailsa Craig' *hp-1* tomato line (obtained from the Tomato Genetics Resource Center, University of California, Davis, CA), as previously described (Engelmann et al., 2010). Tomato cell suspension cultures were continuously maintained in 40 mL of a previously described liquid maintenance media (Robertson, Mahoney, Goodman, & Pavlath, 1995), and were subcultured every 7 days by transferring 2 mL of packed cells and 4 mL of spent media. For CP growth cycles, 4 mL of packed cells with 8 mL of spent media from maintenance cultures were transferred to 80 mL of CP media formulated as previously described (Robertson et al., 1995) and the lycopene cyclase inhibitor, 2-(4-chlorophenyl-thio)triethylamine (CPTA; provided by Betty Ishida, USDA) was filter-sterilized and aseptically added to cultures (0.0745 g/L media) as previously described (Engelmann et al., 2010) on day 1 of a 12 day-long growth period (unless otherwise specified) to stimulate lycopene accumulation. Cultures were wrapped in foil to exclude light and maintained on rotary shakers (Innova 2300, New Brunswick Scientific, New Brunswick, NJ) set to 150 RPM at 25 °C. Culture fresh mass accumulation was measured for all experiments as previously described (Engelmann et al., 2010), and cells and media were reserved and stored at −80 °C for biochemical analyses as needed.

2.2. Serial subculturing

2.2.1. Serial carotenoid production batch culturing trial with unlabeled media

CP media (80 mL) containing glucose (30 g/L) was inoculated with cells and spent media from maintenance cultures, and cells