Journal of Biotechnology 140 (2009) 227–233

Contents lists available at ScienceDirect

Journal of Biotechnology

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

Investigation of cellular targeting of carotenoid pathway enzymes in *Pichia pastoris*

Pyung Cheon Lee [a,b,*], Young geol Yoon [c], Claudia Schmidt-Dannert [d]

[a] *Department of Molecular Science and Technology, Ajou University, Woncheon-dong, Yeongtong-gu, Suwon 443-749, South Korea*
[b] *Department of Biotechnology, Ajou University, Woncheon-dong, Yeongtong-gu, Suwon 443-749, South Korea*
[c] *Department of Laboratory Medicine and Pathology and Institute of Human Genetics, University of Minnesota, 420 Delaware St SE, Minneapolis, MN 55455, United States*
[d] *Department of Biochemistry, Molecular Biology and Biophysics, 1479 Gortner Avenue, University of Minnesota, St. Paul, MN 55108, United States*

ARTICLE INFO

Article history:
Received 5 August 2008
Accepted 21 January 2009

Keywords:
Peroxisome-targeting signal
Pichia pastoris
Carotenoid biosynthetic pathway
Metabolic engineering

ABSTRACT

Cellular targeting of lycopene biosynthetic enzymes was investigated in *Pichia pastoris* X-33. Three lycopene pathway enzymes, CrtE, CrtB, and CrtI, were fused to fluorescent EGFPs with or without a peroxisomal targeting sequence (PTS1) and then expressed in *P. pastoris*. When *P. pastoris* was grown in YPD, the PTS1 fusion enzymes were found to be localized in peroxisomes, whereas the enzymes not fused with PTS1 were equally distributed throughout the entire cell. A similar targeting pattern was also observed in *P. pastoris* strains that were grown in peroxisome-proliferating medium, YPOT. Analysis of the fluorescent images of isolated peroxisomes showed that the PTS1 fused enzymes were dominantly present in peroxisomes whereas small amount of the enzymes not fused with PTS1 were non-specifically sent to peroxisomes. These results indicate that PTS1 specifically target lycopene pathway enzymes into peroxisomes and this targeting pathway was strong enough to overcome their inherent targeting program. In conclusion, we first showed that carotenogenic enzymes can be targeted into the specific cellular location of recombinant hosts and this targeting strategy can serve as the basis for the subsequent development of sophisticated pathway engineering in microorganisms.

1. Introduction

Carotenoids, derivatives of isoprenoids, belong to an important class of natural pigments used in biotechnology applications (Lee et al., 2003). These structurally diverse pigment chemicals have different biological functions including coloration, photoprotection, light-harvesting and precursors for many hormones (Sandmann, 2001; Vershinin, 1999). Currently carotenoids are commercially used as food colorants, animal feed supplements, and more recently, as nutraceuticals, and for cosmetic and pharmaceutical purposes. However, only a few carotenoids can be produced commercially by chemical synthesis, fermentation, or isolation from a few abundant natural sources (Johnson and Schroeder, 1995). The increasing industrial importance of carotenoids has led to renewed efforts to develop bioprocesses for the large scale production of a range of carotenoids, including lycopene, β-carotene, and more structurally diverse carotenoids (Cheng, 2006; Lee et al., 2003).

In most cases, metabolic pathway engineering of prokaryotic microorganisms, such as *Escherichia coli*, does not require a targeting strategy to send heterologously expressed enzymes into specific cellular location. However, one must consider targeting strategies when engineering pathways in eukaryotic microorganisms. Eukaryotes such as yeasts have several cellular organelles such as mitochondria (Yoon et al., 2007) and peroxisomes (Poirier et al., 2002). The organelles, where metabolic activities occur, are physically separated from other cellular components by membrane structures. Therefore, the proper cellular location of heterologously expressed pathway enzymes is important and both cellular and cytoplasmic membranes can be putative locations for membrane-bound enzymes to settle in.

Carotenoid pathway enzymes are known to be membrane-associated or membrane-bound (Britton, 1998). When carotenogenic enzymes are heterologously expressed without a proper targeting system they can be randomly located on the membranes of cellular organelles as well as cytoplasmic membranes. Yeast *P. pastoris* has a cellular organelle peroxisome where farnesyl diphosphate (Fig. 1), an important isoprenoid intermediate for carotenoid biosynthesis (Lee et al., 2005), is known to be generated and present in large amounts (Kovacs et al., 2002). Most of the genetic manipulations used in *P. pastoris* are similar to those used in *Saccharomyces cerevisiae*. Furthermore *P. pastoris*

* Corresponding author at: Department of Molecular Science and Technology, Ajou University, Woncheon-dong, Yeongtong-gu, Suwon 443-749, South Korea. Tel.: +82 31 219 2461; fax: +82 31 219 1610.
E-mail addresses: pclee2000@gmail.com, pclee@ajou.ac.kr (P.C. Lee).

0168-1656/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jbiotec.2009.01.019

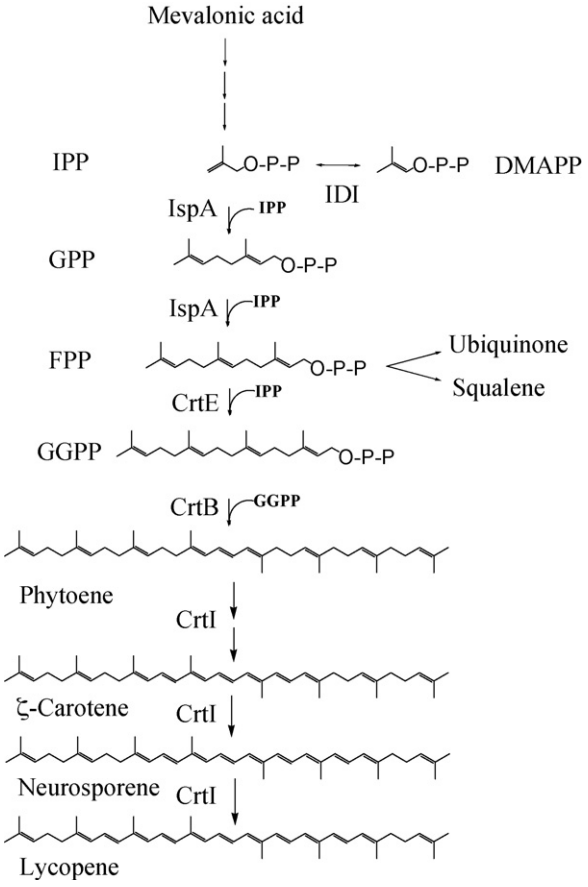

Fig. 1. Simplified engineered lycopene biosynthetic pathway in recombinant *Pichia pastoris*. The enzymes used are IDI, IPP isomerase; IspA, FPP synthase; CrtE, GGPP synthase; CrtB, phytoene synthase; CrtI, phytoene desaturase. The abbreviations used are IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate.

has a strong preference for respiratory metabolism and thus grows to extremely high cell densities without the accumulation or build-up of high concentrations of ethanol as is typically seen in high-density cultures of *S. cerevisiae* (Cereghino et al., 2002). These advantages of *P. pastoris* make this yeast more interesting and viable in the biotechnological production of hydrophobic compounds such as carotenoids.

Although metabolic engineering of *S. cerevisiae* (Verwaal et al., 2007) and *Candida utilis* (Misawa and Shimada, 1997) typically use heterologously expressed carotenogenic enzymes, the cellular location of engineered carotenoid pathway gene products has not yet been examined. Therefore, in this study, we have constructed *E. coli–Pichia* shuttle plasmids expressing carotenogenic enzyme that are fused to fluorescent EGFP with or without the peroxisomal targeting signal 1 (PTS1). We then examined the cellular locations of the fused carotenogenic enzyme in the recombinant *P. pastoris* grown on different carbon sources by fluorescent microscopy.

2. Materials and methods

2.1. Genes and plasmids

E. coli–Pichia pastoris shuttle plasmid pGAPZB and wild type *P. pastoris* X-33 strain were purchased from Invitrogen Corporation (Carslbad, CA). The gene encoding fluorescent EGFP was amplified from pEGFP (Clontech) using 5′ primers that contained an *Eco*RI site at its 5′ end followed by an optimized Kozak consensus (ATGG), a start codon and a 3′ primer containing an *Xba*I site at its 5′ end that was or was not followed by a PTS1 encoded by CAACTTAGA (Table 1). Carotenogenic enzymes fused to EGFP (CrtE_EGFP_n, CrtE_EGFP_p, CrtB_EGFP_n, CrtB_EGFP_p, CrtI_EGFP_n, and CrtI_EGFP_p) were generated by overlapping PCR as follows: first, the genes *crt*E, *crt*B and *crt*I were amplified from pUC-crtE, pUC-crtB and pUC-crtI with a 5′ primer containing an *Eco*RI site at its 5′ end followed by an optimized Kozak consensus, start codon and a 3′ primer covering the C-terminus of the carotenogenic enzymes with an extra sic amino acids (GlyGlyAlaGlyGlyGly) that was used as a spacer instead of stop codon (Table 1). Next, in the 2nd part of the fusion proteins, the gene encoding EGFP, was amplified using 5′ primers that contained the DNA sequence encoding the six amino acid spacer followed by the N-terminus of EGFP and a 3′ primer containing an *Xba*I site at the 5′ end that was or was not followed by PTS1. Finally the PCR products were purified and then used as templates for the 2nd overlapping PCR with the primer sets described above.

Table 1
Oligonucleotides used in this study.

Oligonucleotide	Sequences (5′->3′)
CrtE-F	GGAATTCAAAATGGCAGTCTGCGCAAAA
CrtE-R	GCTCTAGAGCTTAACTGACGGCAGCG
CrtE-PTS-R	GCTCTAGAGCTTACAACTTAGAAC TGACGGCAGCGAG
CrtB-F	GGAATTCAAAATGGCAGTTGGCTCG
CrtB-R	GCTCTAGAGCCTAGAGCGGGCGCTG
CrtB-PTS-R	GCTCTAGAGCCTACAACTTAGAGAGCGGGCGCTGCC
CrtI-F	GGAATTCAAAATGGCACCAACTACGG
CrtI-R	GCTCTAGAGCTCAAATCAGATCCTCCAG
CrtI-PTS-R	GCTCTAGAGCTCACAACTTAGAAATCAGATCCTCCAGC
EGFP-F	CCGGAATTCAAAATGGTGAGCAAGGGCG
EGFP-PTS-R	GCTCTAGATTACAACTTAGACTTGTACAGCTCGTCC
EGFP-R	GCTCTAGATTACTTGTACAGCTCGTCC
fEEGFP-O5	CGGAATTCAAAATGGCAGTCTGCGCAAAA
fEEGFP-I3	GCCACCTCCTGCTCCACCACTGACGGCAGCGAG
fEGFP-I5	GGTGGAGCAGGAGGTGGCATGGTGAGCAAGGGCG
fBEGFP-O5	CGGAATTCAAAATGGCAGTTGGCTCG
fBEGFP-I3	GCCACCTCCTGCTCCACCGAGCGGGCGCTGCC
fEGFP-I5	GGTGGAGCAGGAGGTGGCATGGTGAGCAAGGGCG
flEGFP-O5	CGGAATTCAAAATGGCACCAACTACGG
flEGFP-I3	GCCACCTCCTGCTCCACCAATCAGATCCTCCAGC
fEGFP-I5	GGTGGAGCAGGAGGTGGCATGGTGAGCAAGGGCG

All ligations were carried out with the T4 DNA ligase (New England Biolabs, Beverly, MA) using standard methods. The ligation reaction mixtures were directly transformed into *E. coli* JM109 using chemical transformation. Successful transformants were selected for on Luria–Bertani media (LB) (0.5% yeast extract, 1% tryptone and 0.5% NaCl) plates containing 50 μg/ml zeocin. Plates were incubated overnight at 37 °C and colonies were picked and cultured overnight in LB media containing 50 μg/ml zeocin.

2.2. Construction of plasmids expressing fusion enzymes

PCR products containing the EGFP gene with or withoug PTS1 were ligated into the *Eco*RI–*Xba*I sites of pGAPZB, resulting in pGAPZB_EGFP_n and pGAPZB_EGFP_p, respectively (Table 2). Similarly, the carotenogenic genes fused to EGFP with or without PTS1 were ligated into the *Eco*RI–*Xba*I sites of pGAPZB, resulting in six plasmids: pGAPZB_E_EGFP_n, pGAPZB_E_EGFP_p, pGAPZB_B_EGFP_n, pGAPZB_B_EGFP_p, pGAPZB_I_EGFP_n, and pGAPZB_I_EGFP_p, respectively (Table 2). To check if the fused enzymes were functional *in vivo*, pGAPZB_I_EGFP_n and pGAPZB_I_EGFP_p were transformed into recombinant *E. coli* that also expressed pAC_CrtE_CrtB (Lee et al., 2004). The resulting *E. coli* cells were grown in LB containing 50 μg/ml chloramphenicol and 50 μg/ml zeocin as selection markers. The cells were then extracted with acetone and the crude organic extracts were analyzed with HPLC (Lee et al., 2005) to verify the presence of lycopene.