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Purification of recombinant peroxidase from *Thermobifida fusca* IP1 for β-carotene degradation into industrial flavouring agents

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Abstract

Carotenoids are important colored components of food with several essential health benefits. Because of their characteristic fruity aroma, the cleavage products of carotenoids are being used as flavouring agents in the food industry. Enzymes are needed to get cleavage products of carotenoids in order to avoid the very laborious and costly extraction processes of particular aroma compounds, such as α- and β-ionone, directly from their plant sources. So, for an easy and cost effective access to flavouring agents, the role of enzymes is undeniably crucial. The present work was therefore undertaken to isolate an industrially important recombinant peroxidase enzyme to obtain the flavouring agents. *Thermobifida fusca* (accession number KM677184), isolated from manure piles, was found to effectively degrade β-carotene. The gene encoding *T. fusca* extracellular heme-containing peroxidase (*Tfu*DyP) was amplified from genomic DNA of *T. fusca* and was cloned in vector pET24b. *Tfu*DyP was expressed heterologously in *E. coli* (BL21) and a recombinant enzyme was used for the transformation of β-carotene into volatile products which were identified by the GC-MS (Gas Chromatography-Mass Spectrophotometry). The enzyme was found effective as various volatile degradation products of β-carotene, such as β-ionone, β-cyclocitral, β-ionone-5,6-epoxide and dihydroactinidiolide were identified. With these results, we are inclined to conclude that the extracellular peroxidase enzyme obtained from *T. fusca* IP1 has the potential to be used in the food industry. In spite of the various reports on the subject, the use of this particular bacterial strain in the enzyme production is being reported for the first time in the present work.

Introduction

Carotenoids constitute a group of natural pigmented compounds found in all colored fruits, flowers and vegetables. These isoprenoids are synthesised by all photosynthetic organisms such as plants, cyanobacteria, algae, certain non-photosynthetic bacteria and fungi. Chemically, the carotenoids are lipo-soluble tetra-terpenes with condensed isoprenyl units which build a chromophoric system (Britton, 1995).

Carotenoids cleave into various aromatic compounds which make them highly useful in the detergent, food and perfume industries (Winterhalter and Rouseff, 2002). Among the cleaved products are β-damascenone and β-ionone which are of particular interest (Winterhalter and Rouseff, 2002; Sommerburg *et al.*, 2003; Baldermann *et al.*, 2005). In many plant species, several apocarotenoids are formed by the eccentric cleavage of the polyene chains of carotenes and xanthophylls. Laborious and costly extraction procedures for flavor rich compounds from plant sources have led to the designing of alternative approaches (Zorn *et al.*, 2003; Wache *et al.*, 2006; Zelena *et al.*, 2009).

Owing to the rising demand for natural-based consumer products and to the difficulties encountered during organic synthesis, the aroma and fragrance industries are keenly searching for new approaches/ techniques in the production of aromatic molecules. One of the best solutions to this problem is through the biocatalysis performed by microorganisms or enzymes of microbial origin (Shimoni *et al.*, 2003). The biotransformation of carotenoids especially β-carotene can be used as a rational alternative to produce flavor compounds (Uenojo *et al.*, 2007).

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Numerous reports have been published on the degradation of β-carotene into aromatic compounds (Carmen, Eugenia and Sergio, 2015; Ming-Ming, Shu-Lin and Ming-Tao, 2016).

The aim of the present work was therefore to elucidate, the role of recombinant peroxidase from *Thermobifida fusca* in the formation of volatile aromatic compounds of industrial importance.

Materials and methods

Isolation and molecular identification

The isolation and molecular identification of a bacterial strain was done by using different biological techniques. The strain was obtained from manure piles and subjected to molecular identification by extracting its genome. The extracted DNA was subjected to PCR (polymerase chain reaction) to get an amplified fragment of 16S ribosomal DNA. The reaction was carried out by using optimum conditions and the primers 16S -27F (5' - AGAGTTTGATCMTGGCTCAG-3') and 16S-1492R (5'-TACGGYTACCTTGTTACGACTT -3') were used for this purpose. The amplified product was then subjected to further investigation by using different bioinformative tools such as BLAST and Mega-4 which would establish the phylogenic relationship in order to validate the species identification. The sequences were submitted to the gene bank and the accession number was obtained.

TfuDyP gene amplification from genomic DNA of Thermobifida fusca IP1

The *Tfu*DyP gene was amplified by PCR from genomic DNA of *Thermobifida fusca* IP1. The primers used were *Tfu*DyP-F (5′ACCGAACCA GACACGGAGCGGA3′) and *Tfu*DyP-R(5′TCATC CTTCGATCAGGTCCTGTCCC3′).

Expression and purification of recombinant TfuDyP

The gene encoding for *Tfu*DyP was PCR-amplified from *T. fusca* genomic DNA using the primers NdeI-*Tfu*Dyp-F (5′CAT**ATG**ACCGAAC CAGACACGGAGCGGA3′) and XhoI-*Tfu*Dyp-R (5′CTC**GAG**TCATCCTTCGATCAGGTCCTGTC CC3′) The nucleotides for restriction sites of NdeI and XhoI are shown bold and the underlined segment represents the start codon. The *Tfu*Dyp amplicon was digested with NdeI and XhoI, and cloned into the NdeI and XhoI restriction sites of pET24b (Novagen). The *Tfu*DyP was expressed in *E. coli* BL21 (λDE3, Novagen) transformed with pET24b-*Tfu*DyP construct, grown in Luria-Bertani (LB) medium supplemented with Kanamycin (100 μg/mL) at 37°C. The culture was induced at OD 600 value of 0.7 with 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG), and 15 μM hemin was also added at this point. The culture was then kept in incubator for 24 h at 16°C and harvested by centrifugation (6,500 g for 15 min). The harvested cells were suspended in 20 mM sodium phosphate buffer (pH 6.8) at a ratio of 5 mL of buffer per gram of cells, and lysed using an ultrasonic homogeniser (Q500 Sonicator, QSonica, LLC). The cell debris was withdrawn by centrifugation (12,500 g for 90 min, 4°C), and the supernatant was recovered. The recombinant protein with a C-terminal hexa-Histidine tag was purified at 4°C by passing through a HisTrap FF column which provided a single step purification of proteins from homogenised, unclarified cell lysate by immobilised metal ion affinity chromatography (IMAC).

Gel electrophoresis

The protein purity was monitored by SDS-PAGE using 12% polyacrylamide gel stained with 0.1% (w/v) solution of Coomassie Brilliant Blue stain (Sigma). Broad range protein marker (2-212 kDa) (NEB) were used as protein standards for the determination of molecular masses.

TfuDyP catalysed degradation of β-carotene

β-carotene (Sigma) was used as substrate and emulsion (0.01%) was prepared in Tween 40. The *Tfu*DyP-catalysed transformation was conducted with 15 mU of recombinant *Tfu*DyP having 2 μL 20 mM H_2O_2 and 1.0 mg β-carotene in phosphate buffer saline (PBS) (0.01 M phosphate buffer with 0.0027 M KCl, 0.137 M NaCl, and pH 7.4) at 25°C, 250 rpm for 60 min. The *Tfu*DyP activity was measured spectrophotometrically at ambient temperature in 25 mM citrate buffer pH 3.5, containing 35 nM purified enzyme and 100 mM H_2O_2. The oxidation of the following substrates was tested at the indicated wavelength: Reactive Blue 19 (100 μM) at 595 nm ($\varepsilon = 10\ mM^{-1} \times cm^{-1}$, guaiacol (100 μM) at 465 nm ($\varepsilon = 26.6\ mM{-}1 \times cm{-}1$), 2,6-dimethoxyphenol (100 μM) at 470 nm ($\varepsilon = 49.6\ mM^{-1} \times cm^{-1}$), veratryl alcohol (100 μM) at 340 nm ($\varepsilon = 93\ mM^{-1} \times cm^{-1}$) and o-phenylenediamine (100 μM) at 420 nm ($\varepsilon = 31.3\ mM^{-1} \times cm^{-1}$).

Controls were run with all samples, without the addition of enzyme, or with the addition of heat denatured enzyme (Zelena *et al.*, 2009).

Extraction of degradation products from reaction

mixture

The transformed products were extracted from crude using solvent extraction technique. Ethyl acetate and dichloro methane (DCM) were used as organic solvent for the extraction of cleaved products. The fractions were concentrated at low temperature under reduced pressure followed by GC-MS analysis for the identification of transformed products.

Gas Chromatography-Mass Spectrometry

The GC-MS analysis was conducted using a polar as well as a non-polar phase, on a Fisons GC 8000 instrument equipped with a (polyethylene glycol) ZB-Wax (30 m × 0.32 mm i.d., film thickness, 0.25 µm, Phenomenex, Torrance, CA) column connected to a Fisons MD800 mass selective detector and HP5890 series II GC equipped with a ZB-5MS (30 m × 0.32 mm i.d., film thickness, 0.25 µm, Varian, Palo Alto, CA) column connected to an HP quadrupole mass spectrometer 5989, respectively. Both GC-MS instruments were operated under the same conditions at 70 eV in the EI mode over the range of 33-500 amu. Flow rate of the carrier gas(Helium) was kept 3.1 mL/min (polar phase) or 3.3 mL/min (non-polar phase), respectively. Sample volumes of 1 µL were injected, and oven temperature was kept initially at 40°C for 3 min, and was raised later at the rate of 5°C/min to a final temperature of 240°C for the polar phase and a maximum of 280°C for the non-polar phase, and held constant for 10 min. Kovats method was used to calculate linear retention indices (RIs) using *n*-alkanes (C_7-C_{28}) as external reference. The mass spectral identification was done by comparing spectra with databases (NIST, LIBTX and Wiley) as well as by reference standards (β-ionone and β-cyclocitral).

Results

A strain with the ability to degrade β-carotene was isolated from manure piles and was subjected for identification. The strain was molecularly identified as *Thermobifida fusca* IP1 (Gene Bank accession number KM677184). According to literature search, this strain has the capacity to decolorise dye by peroxidase. After focusing the DyP type peroxidase, primers were designed. The *Tfu*DyP peroxidase gene comprising of 1,290 bp was amplified with PCR from genomic DNA of *T. fusca* and later it was heterologously expressed in *E. coli*. The unprocessed precursor *Tfu*DyP peroxidase comprises of 430 amino acids having mass about 46 kDa (van Bloois *et al.*, 2010). The gene encoding for *Tfu*DyP was further cloned into an IPTG-inducible expression plasmid, pET24b. The recombinant *Tfu*DyP was over expressed with a hexa-Histidine tag on C-terminal to facilitate subsequent detection and one step purification. *Tfu*DyP peroxidase was expressed heterologously in *E. coli* cells (BL21) followed by purification of recombinant protein by passing through Ni^{2+}Histrap column. This heterologous over expression generated approximately 3.5 mg of *Tfu*DyP peroxidase from 1 L liquid culture in pure form. Purified protein sample was then analysed by SDS-PAGE followed by protein staining (Fig. 1). *Tfu*DyP is present in two forms as seen on SDS-PAGE (indicated by A and B respectively in Fig. 1) of which the unprocessed precursor protein moved across the gel to a position corresponding to 45 kDa. This corresponded nicely to the theoretically calculated mass of the unprocessed precursor of 46 kDa (van Bloois *et al.*, 2010).

After expression, the next step was to achieve

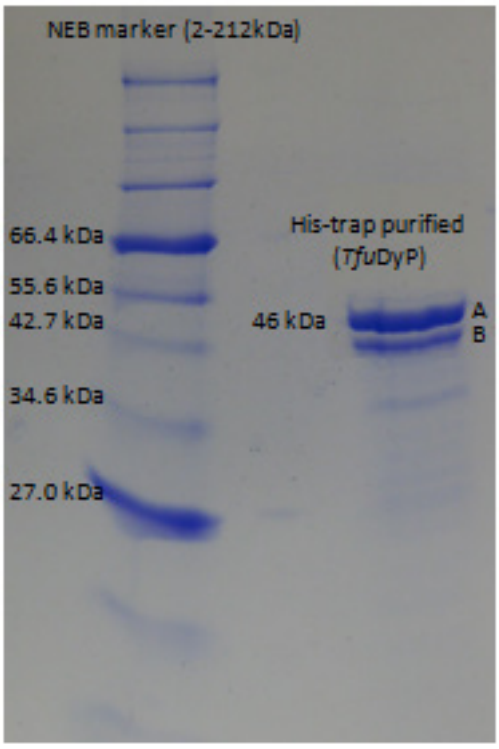

Fig. 1. SDS-PAGE analysis of TfuDyP following purification through His-trap column (A) Precursor and (B) Mature form.

β-carotene catabolism; β-carotene samples were treated with recombinant *Tfu*DyP to obtain the degradation. The biotransformation were performed at pH 4.0, as *Tfu*DyP showed maximum catalytic activity at this pH (Fig. 2).

Although *Tfu*DyP has been reported previously

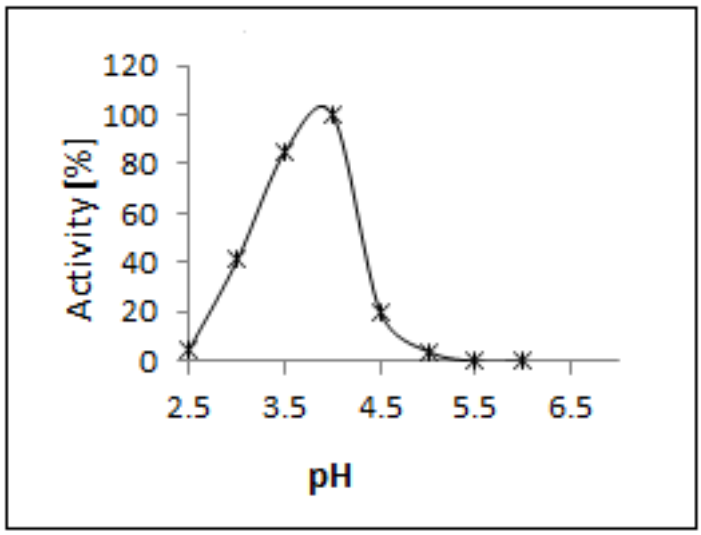

Fig. 2. Effect of pH on carotenoid degradation activity of TfuDy.

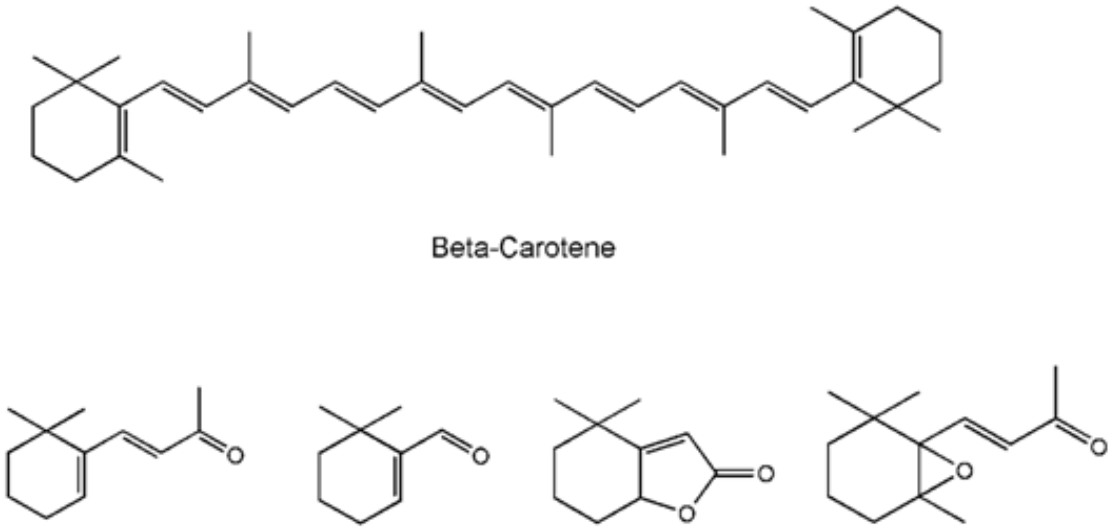

Fig. 3. Chemical compound identified by the GCMS.

to be thermostable even at considerably high temperature of about 60°C (van Bloois *et al.*, 2010), the biotransformation was carried out at 25°C to minimise the losses of the volatile degradation products. The *Tfu*DyP requires H_2O_2 for its catalytic activity. Under these conditions, the substrate was readily transformed within 60 min. The major degradation products detected through the GC-MS analysis comprised of C_{13} nor-isoprenoids and C_{10} products (Figure 3). Controls, without *Tfu*DyP or with heat-inactivated *Tfu*DyP, did not contain any of these degradation products. The GC-MS analysis showed β-ionone, dihydroactinidiolide, β-cyclocitral and β-ionone-5,6-epoxideas volatile degradation products of β-carotene.

Discussion

In the present work, we reported the biodegradation of β-carotene by an environmental bacterial isolate, *T. fusca*. The recombinant peroxidase of this bacterial isolate, *Tfu*DyP, biotransformed the compound *in vitro* to aromatic compounds of industrial importance. GC-MS analysis revealed the identification of β-ionone, dihydroactinidiolide, β-cyclocitral and β-ionone-5,6-epoxideas which are the chief volatile degraded products of β-carotene. In a previous study, β-ionone has been shown as the main metabolite formed in mycelium-free medium of *Lepista irina* (basidiomycete) using β-carotene as a substrate (Zorn *et al.*, 2003). β-ionone is an aromatic compound widely used in fragrance and aroma

industries for its characteristic fruity and sweet smell. It is generated through the direct breakdown of β-carotene, which occurs in plants, flowers and fruits rich in carotenoids (Zorn *et al.*, 2003; Fleischmann *et al.*, 2003; Andrew *et al.*, 2004; Wache *et al.*, 2006; Ibdah *et al.*, 2006; Tieman *et al.*, 2006). A potential mechanism for the carotenoid cleavage between C_9 and C_{10} could start with the abstraction of a hydrogen atom from the allylic methyl group, resulting in a resonance-stabilized carbon radical. Through reaction with oxygen, hydroperoxides may be formed as intermediate. Hock cleavage would yield two carbonyl compounds (Zorn *et al.*, 2003). The formation of β-ionone-5,6-epoxide may occur by the breakdown of β-carotene-5,6-epoxide or alternatively by the epoxidation of liberated β-ionone. β-carotene-5,8-epoxide probably represents the direct precursor of dihydroactinidiolide (Bosser *et al.*, 1995).

Aroma serves as the most indicative and imperative parameter of quality in the acceptance or rejection of a food product. In various natural foods, aromatic compounds also exist either as free volatiles, or in the form of complexes with non-volatile moities such as cystein sulfoxide, cinnamic acid derivatives, carotenoids, glycosides and thioglycosides (Solis *et al.*, 2007).

Although there is a very limited data reported on the degradation of carotenoid moiety substances by peroxidases, carotenoid degradation has been described by some plant peroxidases. Solubilized thylakoid membranes of olives are also reported to exhibit a carotenoid-degrading peroxidative activity (Gandul-Rojas *et al.*, 2004), and some incomplete degradation of β-carotene has been found with horse radish peroxidase, soybean peroxidase and lactoperoxidase (Gelinas *et al.*, 1998). In contrast to plant carotenoid cleavage peroxidases that act highly selectively, this recombinant enzyme of *T. fusca* (*Tfu*DyP peroxidase) catalysed a wider range of volatile products. In addition to the production of "bioflavors", the recombinant peroxidase could become a valuable tool for detergents and food-bleaching purposes (Zorn *et al.*, 2008). The recombinant enzyme of *T. fusca* showed a promising biotransformation capacity for the production of various flavor compounds from β-carotene of industrial importance. New techniques for the biogeneration of natural flavour compounds are likely to be investigated in future and will certainly be a valuable alternative to the chemical synthesis. The consumer's increasing demand for natural products has reinforced the need for the production of these compounds via biotechnology-driven processes.

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