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Hydrophilization of bixin by lipase-catalyzed transesterification with sorbitol

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ABSTRACT

Bixin is one of the most used yellow-orange food colorants in the food industry. The polyene chain of bixin makes it highly hydrophobic and less suitable for water-based food formulations. Lipase-catalyzed reactions of bixin with sorbitol were studied to synthesize a new derivative of bixin with potential hydrophilic properties. Interestingly, we show that the lipase-catalyzed reaction of bixin leads to a transesterification reaction and formation of a transesterified product, sorbitol ester of norbixin (SEN). The reaction efficiency was optimized with various immobilized lipases at different water activity levels in the organic solvent, 2-methyl-2-butanol. Among the examined lipases, immobilized *Candida antarctica* lipase B (Novozyme 435) provided the highest reaction yield at a water activity close to zero. Tetrahydrofuran (THF) was used as co-solvent to improve bixin solubility. The optimization of the reaction conditions with 20% THF lead to a total reaction yield of 50% of SEN.

1. Introduction

The annatto colorant is one of the most widely used natural colorants in the food industry. The colorant is extracted from the seeds of the tropical shrub, *Bixa Orellana*. The major color components of annatto extract are bixin and norbixin (Scotter, Wilson, Appleton, & Castle, 1998). The bixin structure contains a long polyunsaturated hydrocarbon chain with one carboxylic acid and one methyl ester group in each end of the chain, which results in a high degree of lipophilicity. Norbixin is mainly produced by saponification of bixin in alkaline solution to form the diacid derivative of bixin. Norbixin has better hydrophilic properties, which makes it more applicable for particular food products such as cheddar cheese (Kang, Campbell, Bastian, & Drake, 2010).

The food industry lacks stable natural hydrophilic colorants in the range of yellow–red, which can be used in low pH water-based food products such as beverages. As implied above, bixin is not soluble in aqueous food items. Furthermore, norbixin, which has been known as the water soluble annatto colorant is only soluble in alkaline solutions and precipitates in acidic and neutral conditions (Breukers, Øpstad, Sliwka, & Partali, 2009).

One way to increase the water solubility of bixin could be by appending a hydrophilic molecule to bixin to increase its polarity and thereby improve the aqueous solubility. There is a lack of studies investigating the reaction of bixin with hydrophilic compounds. To our knowledge, only one paper concerning the modification of the bixin scaffold by enzymatic reaction with L-ascorbic acid has been reported (Humeau, Rovel, & Girardin, 2000). Humeau et al. suggested that the reaction of bixin with ascorbic acid can occur via the ester bond to form a new bixin derivative. Besides, as ascorbic acid is an antioxidant, it might protect bixin from heat or light induced degradation. The enzymatic reaction was performed with immobilized lipase from *Candida antarctica*. According to Humeau et al. the conversion ratio of ascorbic acid to ascorbyl ester of norbixin was only 25% after 144 h reaction. It was assumed that the low reaction yield might be due to the low solubility of substances in the reaction solvent 2-methyl-2-butanol (2M2B). Solvents such as tert-butanol and diacetone alcohol were examined, but none of them improved the solubility of bixin (Humeau

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et al., 2000).

Hydrophilic derivatives of bixin can be synthesized through esterification or transesterification reactions using biocatalysts. Lipases are widely used as the biocatalyst for esterification or transesterification reactions and the immobilized ones have been shown to exhibit a better catalytic activity compared to the non-immobilized lipases in non-aqueous media (Adlercreutz, 2017). The use of organic solvents in enzymatic reactions can favor an esterification or transesterification reaction on bixin rather than a hydrolysis, to prevent the formation of norbixin.

Among the most commonly used non-immobilized lipases for esterification or transesterification reactions are those from the microorganisms *R. arrhizus, C. rugosa* and *P. cepacia.* Immobilized lipases, such as Novozyme 435 (*C. antarctica* lipase B, CALB), Lipozyme RM-IM (lipase from *R. miehei*) and Lipozyme TL-IM (lipase from *T. lanuginosus*) are also commercially available.

Although a lower water content is favorable for the esterification or transesterification reactions, we need to take into account that the activity of lipases are highly dependent on their hydration level. A low water content has also been shown essential in lipase-catalyzed reactions in non-aqueous solutions and the water content of the reactions can significantly impact the catalytic activity of lipases (Adlercreutz, 2013; Klibanov and Zaks, 1987). Water is known to act as a molecular lubricant for lipase in the non-aqueous media, whereas if the water layer is stripped away, the active site of the lipase might show conformational changes and thus display lower catalytic activity (Halling, 2004).

Lipases respond differently to the amount of water in organic media. Some retain good activity at low water activity, e.g. the lipase from *R. arrhizus* and CALB, while some require higher water activity level to show good catalytic activity, e.g. lipase from *P. cepacia* (Wehtje & Adlercreutz, 1997). It is difficult to measure the amount of water bound to the lipase, but the water activity level of the reactions can be controlled and adjusted by different saturated salt solutions (Greenspan, 1977).

In the present study, we aim to modify the chemical structure of bixin by introducing sorbitol as a hydrophilic compound into the bixin scaffold, using a lipase-catalyzed reaction. Sorbitol has previously been used to synthesis a derivate of astaxanthin in order to improve the water solubility (Lockwood et al., 2005). Moreover, we will investigate whether different lipases will favor the esterification or the transesterification reaction. Furthermore, we will find the optimal water activity level for the individual lipases and reaction solvents, in order to optimize the reaction yield.

2. Materials and methods

2.1. Reagents

Bixin was provided by Chr. Hansen A/S (Taastrup, Denmark). Lipase acrylic resin from *Candida antarctica*, ≥5,000 U/g (Novozyme 435), Lipozyme RM-IM (lipase from *R. miehei*) and Lipozyme TL-IM (lipase from *T. lanuginosus*), were purchased from Sigma-Aldrich. Non-immobilized lipases, *Rhizopus arrhizus, Candida rugosa* and *Pseudomonas cepacia* were purchased from Sigma-Aldrich. Porous polypropylene (Accurel MP 1000) was purchased from Membrana GmbH (Obernburg, Germany). *P*-nitrophenyl butyrate (*p*-NPB, 98% pure), D-Sorbitol (≥98% pure) and all other solvents were of analytical grade. Molecular sieve (3 Å, 2 mm bead, 10 mesh) were purchased from Merck (Darmstadt, Germany). Silica gel (60 Å, 70–230 mesh, 63–200 μm) were purchased from Sigma-Aldrich.

2.2. Lipase immobilization

Lipases from *R. arrhizus, C. rugosa* and *P. cepacia* were immobilized separately on polypropylene with particle size smaller than 500 μm. Ethanol (0.5 mL) was added to 0.5 g of solid support, polypropylene MP 1000 and mixed thoroughly. Lipase (0.5 g) was dissolved in 10 mL phosphate buffer (50 mM, pH 7.0) and mixed and centrifuged at 2700*g* for 5 min to remove undissolved particles. Subsequently, the supernatant was mixed with the prepared polypropylene support solution. The immobilization was carried out by shaking the solution using an orbital shaker over night at room temperature. The preparations were filtered and gently washed with phosphate buffer (50 mM, pH 7.0) and then dried in a desiccator under vacuum overnight. The immobilization yield was determined by measuring the enzymatic activity of the enzyme solutions before and after immobilization based on the hydrolysis of *p*-nitrophenyl butyrate (Gitlesen, Bauer, & Adlercreutz, 1997).

2.3. Equilibration of water activity

The thermodynamic water activity level of all experiments were adjusted by equilibrating the substrates and solvents with aqueous saturated salt solutions in separated vessels over two days. Equilibration was obtained using $MgCl_2$ and $Mg(NO_3)_2$ at room temperature to reach the water activity levels of 0.33 and 0.53, respectively (Greenspan, 1977). The molecular sieve was used to dry solvents to reach a water activity level of zero. The substrates were dried under vacuum.

2.4. Synthesis of sorbitol ester of norbixin

Bixin (59 mg, 50 mM), sorbitol (27 mg, 50 mM) and immobilized lipase 12 mg (20% of the total weight of bixin) were weighed into a 4 mL septum capped vial. A mixture of 2M2B:THF (3 mL) was added in different ratios based on reaction conditions from 0 to 100% of THF. The reaction mixture was shaken using a MHR11-TH21 thermo shaker (HLC, Germany) at 58 °C and 750 rpm. After 24 h of reaction, the lipase was separated from the reaction mixture by centrifugation (centrifuge model 4417R, Hamburg, Germany) at 20,200*g* for 10 min. The supernatant was separated and stored at −20 °C until further analysis using ultra-High Performance Liquid Chromatography (uHPLC) with reverse phase separation.

2.5. Purification

The SEN was isolated by glass column chromatography. The column was prepared with silica gel using a mixture of chloroform/methanol/water/acetic acid 86:12:1:1 (v/v/v/v) as elution solvent. The fractions containing the product were combined, the solvent was evaporated using a vacuum centrifuge and the residue was analyzed and quantified by uHPLC.

2.6. Thin layer chromatography analysis

Thin layer chromatography (TLC) analysis was used to monitor the reaction progress. The reaction mixtures were diluted in THF 1:50 (v/v) and analyzed on TLC plates (TLC Silica gel 60, 5 × 7.5 cm, Merck, Germany) with the elution system chloroform/methanol/water/acetic acid 86:12:1:1 (v/v/v/v). Under these conditions, the retention factors (R_f) were 0.19, 0.62 and 0.81for SEN, norbixin and bixin, respectively.

2.7. Quantification

Quantification was performed on an uHPLC Shimadzu LC-2040C 3D system (Duisburg, Germany), equipped with a photodiode array detector (PDA). The analyte mixture was diluted 50 times in THF and 2 μL was injected onto a C18 column (2.6 μm, 100 mm, 4.6 mm, Kinetex), at 1.6 mL/min flow rate and a column temperature of 35 °C. The mobile phases consisted of solvent A with 2% aqueous acetic acid and solvent B with acetonitrile with 0.2% acetic acid. The step linear gradients were; 45% B at 0 min, 70% B at 4 min, 70% B at 9 min, 100% B at 10 min, 100% B at 14 min, 45% B at 15 min and 45% B at 20 min. Detection was