Biotechnology Letters (2006) 28: 581–585
DOI 10.1007/s10529-006-0019-2

Enzymatic synthesis of cinnamic acid derivatives

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Received 13 December 2005; Revisions requested 10 January 2006; Revisions received 17 January 2006; Accepted 18 January 2006

Key words: Cinnamic acid, ethyl ferulate, ferulic acid, octyl methoxycinnamate, Novozym 435

Abstract

Using Novozym 435 as catalyst, the syntheses of ethyl ferulate (EF) from ferulic acid (4-hydroxy 3-methoxy cinnamic acid) and ethanol, and octyl methoxycinnamate (OMC) from *p*-methoxycinnamic acid and 2-ethyl hexanol were successfully carried out in this study. A conversion of 87% was obtained within 2 days at 75 °C for the synthesis of EF. For the synthesis of OMC at 80 °C, 90% conversion can be obtained within 1 day. The use of solvent and high reaction temperature resulted in better conversion for the synthesis of cinnamic acid derivatives. Some cinnamic acid esters could also be obtained with higher conversion and shorter reaction times in comparison to other methods reported in the literature. The enzyme can be reused several times before significant activity loss was observed.

Introduction

In addition to their antioxidant activity, some naturally occurring phenolic acids are known to display various biological functions which are mainly related to the modulation of carcinogenesis. A major portion of the antioxidant activity of oil-seeds is attributed to phenolic acids, including hydroxylated (*p*-coumaric acid, caffeic acid) or methoxylated (ferulic acid) derivatives of cinnamic acids. Octyl methoxycinnamate (OMC) is a UV-B absorbing compound that is widely used as a non-allergic sunscreen. Ethyl ferulate (EF) can prevent autooxidation of model substrates more effectively by extending the induction time of this process (Kikuzaki *et al.* 2002).

However, the hydrophilic character of phenolic acids reduces their antioxidant effectiveness in inhibiting autooxidation of fats and oils. This has been reported as serious disadvantage if an aqueous phase is also present (Schuler & Hudson 1990). The strategy of esterification of hydrophilic compound with aliphatic molecules, such as fatty alcohols, can be employed to alter their solubility in oil-based formulae and emulsions. Chemical synthesis of these esters is difficult as phenolic acids are heat-sensitive and susceptible to oxidation under certain pH conditions (Pyysalo *et al.* 1997). Enzymatic esterifications of phenolic acids with aliphatic alcohols have been reported (Guyot *et al.* 1997, Stamatis *et al.* 1999, Priya & Chadha 2003, Topakas *et al.* 2003, Hatzakis *et al.* 2003). However, all these researches failed to achieve good results within a reasonably short reaction time.

The goal of this study is to synthesize EF and OMC by the enzymatic esterification of ferulic acid and *p*-methoxycinnamic acid, respectively. The molecular structures of *p*-methoxycinnamic acid, OMC, ferulic acid and EF are shown in Figure 1.

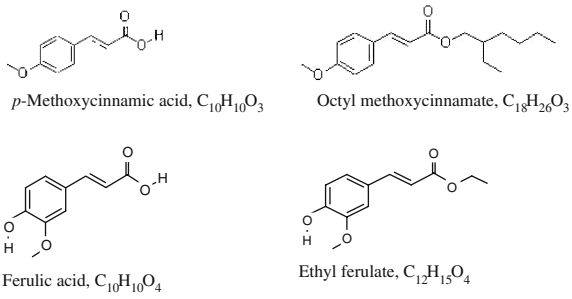

Fig. 1. Molecular structure of reactants and products in this study.

Materials and methods

Materials

Novozym 435 (*Candida antarctica* lipase immobilized on acrylic resin) was a gift sample from Novo Nordisk (Denmark). It has a specific activity of 7 PLU (propyl laurate units)/mg based on ester synthesis. Ferulic acid and *p*-methoxycinnamic acid were purchased from Sigma and Acros, respectively. All solvents and reagents were either of HPLC grade or AR grade. All other chemicals used were obtained from commercial sources.

Esterification reactions

Typically, *p*-methoxycinnamic acid (30 mg) and 2-ethyl hexanol (210 mg) in 1 ml isooctane were put in a 10 ml sealed vial and incubated at 80 °C in an oil bath without stirring. For the synthesis of ethyl ferulate, ferulic acid (30 mg) and ethanol (50 μl) in 3 ml solvent were incubated at 75 °C. The solvent used was mostly isooctane unless in some cases where the effect of types of solvent was investigated. Reactions were initiated by the addition of Novozym 435. Progresses of the reaction were monitored by HPLC. The effect of stirring was investigated and it was found that stirring had negligible effect on the syntheses of EF and OMC.

HPLC analysis

Samples were analyzed by an HPLC device (Jasco, model PU980) equipped with a variable-wavelength UV detector. A Luna 5 μ C18 (250 × 4.6 mm, Phenomenex) column was used at room temperature. The mobile phases for OMC and EF analyses consisted of methanol/acetic acid (100:0.01, v/v.) and acetonitrile/water/acetic acid (80:20:0.01, by vol.), respectively, at 0.5 ml/min. Detection was at 325 nm.

Purification of the product

After the completion of reaction, lipase was filtered off using filter paper (Advantec filter paper, type no. 2, Japan). The enzyme was washed thoroughly with hexane and used in the reusability study. The filtrate was concentrated by drying under vacuum in a rotary evaporator, and the target product was purified by column chromatography using hexane/ethyl acetate (96:4, v/v) as the solvent. A 400 mm×20 mm glass tube was used as the chromatography column with a valve to control the flow-rate of eluent. Silica gel (100–200 mesh), 25 g, was activated for 60 min at 120 °C. Slurry of silica gel in the hexane-ethyl acetate solvent system was poured into a column previously half filled with the solvent. A slight flow of the solvent was allowed during packing. The solvent level was lowered until it was 1 cm above the stationary phase. The exit of the chromatography column was plugged with glass wool to retain the solids. About 30 mg of the crude product obtained from the synthesis of EF or OMC was dissolved in the solvent (2 ml). A small amount (0.3 ml) of this solution was applied to the chromatography column. The column was eluted with the solvent at 1–2 ml/min. The eluates were collected and analyzed by HPLC.

Results and discussion

The effect of solvent

Log *P*, where *P* is the partition coefficient of solvent between octanol and water, is a parameter which is a measure of hydrophobicity of a solvent. Figure 2 shows the effect of Log P on the conversion in the lipase-catalyzed synthesis of OMC. As shown in Figure 2, isooctane, having the highest Log P, gives the highest conversion

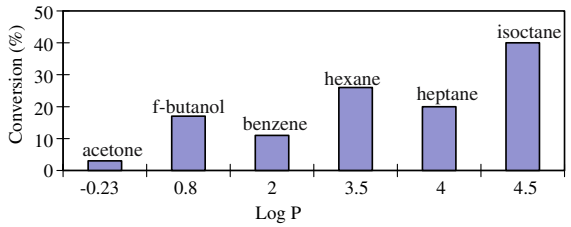

Fig. 2. The effect of solvent on the synthesis of OMC. Reactions were carried out in 3 ml solvent containing 30 mg *p*-methoxycinnamic acid, 210 mg 2-ethyl hexanol and 60 mg Novozym 435 at 65 °C for 24 h. Conversions were calculated as w/w ratio of *p*-methoxycinnamic acid reacted to its initial amount.

after 24 h of reaction. Therefore, isooctane was chosen as the solvent in this study.

The effect of temperature on the syntheses of OMC and EF

The reaction rate increases with increasing temperature. However, high reaction temperature may cause deactivation of enzyme. Figures 3 and 4 show that to reach 90% conversion, the reaction took 24 h for OMC synthesis at 80 °C and 48 h for EF synthesis at 75 °C, respectively. According to Novozym 435 product sheet from Novo Nordisk, Novozym 435 is a heat-tolerant, immobilized enzyme with a maximum activity at 70–

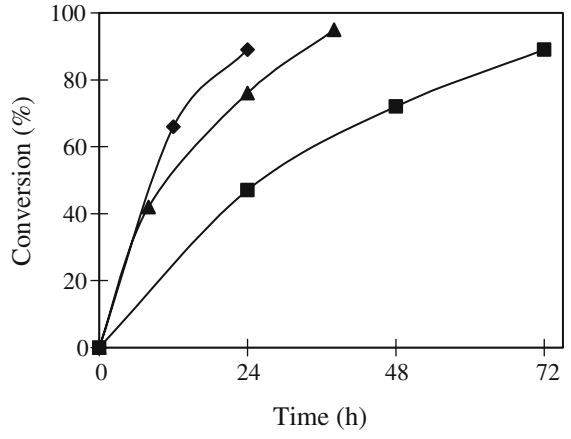

Fig. 3. Effect of reaction temperature on the synthesis of OMC: (♦) 80 °C, (▲) 70 °C, (■) 65 °C. Reaction conditions: reaction was carried out in 1 ml isooctane containing 30 mg *p*-methoxycinnamic acid, 210 mg 2-ethyl hexanol and 60 mg enzyme. Conversions were calculated in the same way as in Figure 2.

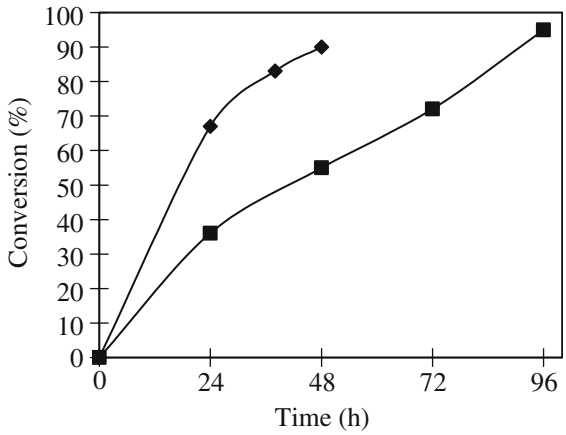

Fig. 4. Effect of reaction temperature on the synthesis EF: (♦) 75 °C, (■) 65 °C. Reaction conditions: reaction was carried out in 3 ml isooctane containing 30 mg ferulic acid, 0.3 ml ethanol and 60 mg enzyme. Conversions were calculated as w/w ratio of reacted ferulic acid to its initial amount.

80 °C. However, it is suggested that the enzyme should be used at 40–60 °C for the sake of its stability. In this work, the reusability study of Novozym 435 was carried out at 80 °C for OMC synthesis and 75 °C for EF synthesis and the results will be discussed later.

The effect of water content

Since water is a product in esterification reaction, its concentration should be kept as low as possible in order to shift the thermodynamic equilibrium in favor of product formation. However, a small amount of water is essential for maintaining the three dimensional structure of protein that is crucial to the catalytic function of enzyme (Timasheff 1993). Several investigators have reported that a certain amount of water is essential to maintain both thermal stability (Volkin *et al.* 1991) and catalytic activity (Zaks & Klibanov 1988) of enzymes in non-aqueous media. Under the experimental conditions used in this study for the synthesis of EF, theoretically 2.7 mg water was generated after the completion of reaction. A small amount of water (1 mg) added to the mixture at the beginning of the reaction had no effect on the reaction. However, conversion does decrease if more water is added (data not shown). A similar phenomenon was observed on the effect of added water on the synthesis of OMC.

The effect of substrate to enzyme ratio

Recently, the direct esterifications of phenolic acid (including cinnamic acid derivatives) with aliphatic alcohol catalyzed by *Candida antarctica* and *Rhizomucor miehei* lipases in anhydrous organic solvents or solvent-free systems were reported (Guyot *et al*. 1997, Stamatis *et al*. 1999, Priya & Chada 2003, Lue *et al*. 2005). Most of these investigations reported low yield, long reaction time and inactivation of lipase. These were mainly the results of using polar solvent and low reaction temperature in their studies.

In this work, some commercially available lipases either in free or immobilized forms were screened for their activity in producing the target compounds. Preliminary results showed that Novozym 435 possesses reasonable activity toward the synthesis of target compounds (data not shown). Therefore, Novozym 435 was employed as the biocatalyst in this study.

The effect of enzyme to acid weight ratio on the initial rate of esterification of OMC was investigated. As enzyme to acid ratio increases from 1:1 to 2:1, the initial rate increases rapidly from 0.7 to 2 mM/h and then increases slowly to 2.4 mM/h as the enzyme to acid ratio increases to 3:1. When higher enzyme concentration is used, more active sites of the enzyme will be available for the binding of substrates which leads to the increasing of reaction rate. At fixed substrate concentration, further increase in enzyme concentration will not give significant increase in reaction rate since no more substrate molecules are available for binding onto the active site of the enzyme. In this study, an enzyme to acid ratio of 2:1 was employed.

The effect of substrate ratio

Some alcohols, such as methanol or ethanol, can inactivate enzymes (Soumanou & Bornscheuer 2003). The effect of step-wise addition of ethanol on the esterification of ferulic acid was therefore investigated. The reaction was carried out at 70 °C with 30 mg ferulic acid and 60 mg Novozym 435 in 3 ml isooctane with 3 μl ethanol added every 12 h. Approximately 93% conversion was obtained after 3 days. If 12 μl ethanol was used instead of 3 μl every 12 h, the same conversion was obtained in 2.5 days. Since *ca*. 9 μl ethanol is stoichiometrically required for every 30 mg ferulic acid, this demonstrates that excess alcohol is beneficial to the formation of the product. Further studies showed that, instead of stepwise addition, adding 50 μl ethanol at the beginning of the reaction gave *ca*. 90% conversion in 2 days. Therefore, stepwise addition of ethanol is not needed in the synthesis of ethyl ferulate catalyzed by Novozym 435.

The effect of molar ratio of ferulic acid to ethanol on the synthesis of ethyl ferulate was investigated and it was found that a ferulic acid to ethanol molar ratio of 1:5 gave the highest conversion (data not shown). Therefore, this value was employed in the synthesis of EF in this study.

The effect of phenolic acid structure

Experimental results obtained in this study indicate that maximum conversion in the OMC synthesis can be attained in shorter reaction time than that in the EF synthesis. Guyot *et al*. (1997) first pointed out the electron donating effect in cinnamic acid ester synthesis. The electron donating effects intrinsically deactivate the electrophilic carbon center of the carboxylic group for nucleophilic attack of the alcohol. Stamatis *et al*. (1999) reported that the hydroxyl-substituted cinnamic and benzoic acid derivatives (especially *ortho*- and *para*-isomers) inhibited the catalytic action of lipase. For OMC synthesis, because there is no OH group on the benzene ring, the electron donating effect will not occur. Therefore, the reactivity of *p*-methoxycinnamic acid is higher than that of ferulic acid which has an OH group on the *para*-position.

Reusability of the enzyme

The concentration of 2-ethyl hexanol plays an important role on the reusability of enzymes in the synthesis of OMC. As shown in Figure 3, at 80 °C and with isooctane as the solvent, the conversion reached 90% after 24 h. Figure 5 shows that Novozym 435 was very stable in the synthesis of OMC when isooctane was employed as the solvent. The enzyme can be reused 8 times before significant activity loss was observed. When isooctane was replaced by 2-ethyl hexanol, the conversion decreases drastically in the reusability study of the enzyme (data not shown).

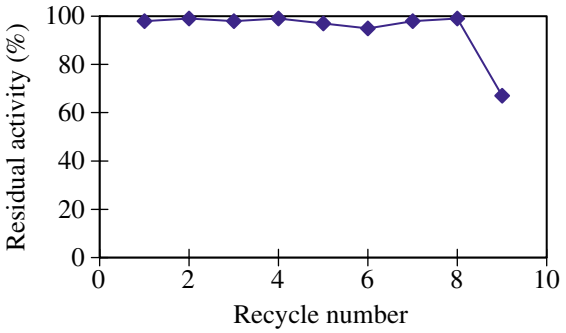

Fig. 5. Reuse stability of the immobilized lipase on the synthesis of OMC. Reaction was carried out in 1 ml isooctane containing 30 mg *p*-methoxycinnamic acid, 210 mg 2-ethyl hexanol and 60 mg enzyme at 80 °C for 24 h. Conversions were calculated in the same way as in Figure 2.

A reusability study was also conducted in the synthesis of EF. It was found that Novozym 435 can only be reused three times and after that the ester conversion dropped to lower than 90% (data not shown). The substantial loss of lipase activity in this case is attributed to the use of ethanol as one of the reactant. In the synthesis of OMC, the alcohol used was 2-ethyl hexanol, a branched medium chain alcohol. In the synthesis of EF, a more polar and hydrophilic solvent, i.e. ethanol was used. The significant loss of lipase activity in the synthesis of EF may be caused by the existence of ethanol near the immobilized enzyme particle that distorts the essential water layer, which is essential for enzyme activity.

Acknowledgements

The financial support of National Science Council of Taiwan under grant NSC92-2214-E011-003 is gratefully acknowledged.

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