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Double-lipase Catalyzed Synthesis of Kojic Dipalmitate in Organic Solvents

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Abstract Kojic dipalmitate(KDP) was synthesized by the way of esterification of palmitic and kojic acids in organic solvent, with double-lipase as the biocatalyst. Four commercially available lipases(Amano PS, Novozym 435, Lipozyme TL IM and Lipozyme RM IM) were used to group six double-lipase combinations. These combinations were studied and Amano PS-Novozym 435 was found to have the best efficiency and was selected for optimizing the reaction conditions. The optimal reaction conditions were as follows. The mass ratio of Amano PS to Novozyme 435 was 1:1.5, with stirring speed of 500 r/min, substrates molar ratio of 1:2, 50 °C, 5%(mass fraction) catalyst dosage of kojic acid quantity and using acetone as co-solvent. Under these conditions, the diesterification of C5 and C7 hydroxyl groups of kojic acid molecule could be well catalyzed by double-lipase and realize a high KDP yield of 85%.

Keywords Kojic acid; Palmitic acid; Kojic dipalmitate; Double-lipase catalysis; Diesterification

1 Introduction

Kojic acid[5-hydroxy-2-(hydroxymethyl)-1,4-pyrone] is a cheap fungal metabolite produced by many species of *Aspergillus* and *Penicillium*[1]. It is well known as one of the most popular tyrosinase inhibitors and has been widely used as a whitening or anti-browning agent[2—5]. However, kojic acid is water-soluble and unstable at high temperatures, prohibiting it from being directly incorporated in oil base cosmetic products. To improve the properties of kojic acid, such as storage stability, compatibility and oil-solubility, many kojic acid derivatives, including kojic acid-tripeptides, kojic acid glucoside, kojic acid esters, and so on, have been synthesized[6—11]. Some kojic acid derivatives were approximately 15 times more stable than kojic acid[7].

Kojic dipalmitate(KDP) can be hydrolyzed by esterases in skin cells and release kojic acid *in situ*[10], and has been used in cosmetics. The esterification method of kojic acid with long chain fatty acids in the presence of acid or alkaline catalysts usually results in a complex mixture[12]. The industrial production of kojic dipalmitate is performed in a chemical way, which usually uses palmitoyl chloride as raw material, and pyridine as catalyst. The method has several disadvantages, such as high equipment requirements, complicated procedures for product purification and environmental pollution because of the use of organic solvent[13]. Recently, lipase-catalyzed reaction has been used for the acylation of kojic acid. Many long chain fatty acids, such as lauric, oleic and ricinoleic acids, have been used as acyl group supplier for the esterification using lipases as the biocatalyst. However, to the best of our knowledge, there are no studies on the enzymatic diesterification of kojic acid with palmic acids. One of the important reasons is that there are two hydroxy groups, C5 hydroxyl group and C7 hydroxyl group(Scheme 1), in kojic acid molecule. The esterification of C5 hydroxyl group could be carried out easily[14—16]. But little is reported about the esterification of C7 hydroxyl group. The reaction can only be realized at special conditions. For example, in solvent-free system, the fatty acid has the ability of lipase regioselective esterification of diols[12,17,18] that only one of its hydroxyl group can be esterified. Therefore, it will be very difficult to prepare kojic dipalmitate by lipase-catalyzed esterification.

There are many reports about the esterification of kojic acid with single enzyme as the biocatalyst, but only one —OH(C5 hydroxyl group or C7 hydroxyl group) can be esterified. The esterification of C5 hydroxyl group was performed, and lipase from *Pseudomonas cepacia* showed the highest specific activity among the enzymes tested[15]. Whole cell of mutated *Xanthomonas campestris* was successfully used as biocatalyst for the glucosylation of kojic acid[8]. To improve its lipophilic properties for better application in the oil-based cosmetics, Liu *et al*.[14] used *Pseudomonas cepacia* lipase and *Penicillium camembertii* lipase to catalyze the esterification of kojic acid to synthesize kojic acid monolaurate and kojic acid monooleate. Kojic acid monoricinoleate was successfully synthesized by lipase-catalyzed esterification of ricinoleic and

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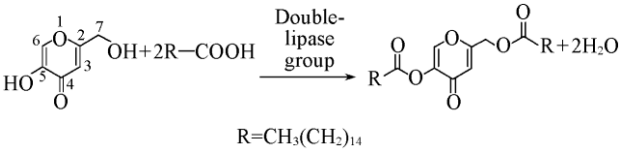
kojic acids in solvent-free system using three-immobilized-enzyme system[12].

In this study, kojic dipalmitate was synthesized using double-lipase group as the biocatalyst in organic solvents (Scheme 1). Catalytic activities of different double-lipase combinations were evaluated. For the selected lipases combination, reaction conditions were optimized, such as the organic solvents, enzyme proportion, stirring speed, reactant molar ratio, temperature and catalyst concentration.



Scheme 1 Synthesis of kojic acid dipalmitate

2 Experimental

2.1 Materials

Lipase from *P. cepacia*(Amano PS) was purchased from Amano International Enzyme Co.(Nagoya, Japan). Immobilized thermostable lipase from *Candida antarctica*(Novozym 435), Lipozyme TL IM from *Thermomyces lanuginosus* and Lipozyme RM IM from *Rhizomucor miehei* were purchased from Novo Nordisk Inc.(Danbury, CT). Acetone, acetonitrile, chloroform, cyclohexanol, diethyl ether, ethyl acetate, *tert*-butyl alcohol, tetrahydrofuran and toluene were obtained from Merck Chemical Co.(Darmstadt, Germany). Kojic acid and palmitic acid were purchased from Sigma Chemical Co. All other chemicals used were of reagent grade.

2.2 Equipments and Analytical Methods

Reactions were performed in a batch stirred 250-mL reactor, equipped with an agitator, a temperature controller and a reflux unit. The reaction temperature was precisely controlled by conducting the experiments in a thermostatic oil bath with an electrical device connected to a PID controller which limits the temperature oscillation within ±0.1 °C.

A Hitachi LC system equipped with a Hitachi L-7100 high pressure pump and a Hitachi L7420 diode array detector (DAD)(Tokyo, Japan) was employed. A computer equipped with an LC System Manager software connected to the LC system was used to process all chromatographic data. A PLGel Mixed-D(polystyrene/divinylbenzene co-polymer)(300 mm× 7.5 mm i.d., 5 μm) analytical column from Polymer Laboratories(Church Stretton, Shropshier, UK) and a PLGel Guard (50 mm×7.5 mm i.d., 5 μm) guard column made up of the same stationary phase were used in the proposed method. The analytical procedures and the operating conditions were according to the method described by Balaguer *et al*.[19].

^{1}H NMR and ^{13}C NMR were recorded on a Bruker DRX500(500 MHz) instrument. The chemical shifts were expressed relative to tetramethylsilane(TMS), and the coupling constants *J* were given in hertz(Hz). Mass spectra were recorded on a Mariner ESI-TOF mass spectrometer(HRMS). Melting points were determined with a WRR melt apparatus. Mass spectra were recorded on a Mariner ESI-TOF mass spectrometer.

2.3 Lipase-catalyzed Synthesis of Kojic Dipalmitate

Organic solvent, kojic acid and palmitic acid were firstly evenly mixed in the reactor. When the set temperature reached, the lipases were added. The samples were taken at regular intervals and analyzed by HPLC. The product was purified by a silica gel 60(mesh size 40—63 mm) column with petroleum ether(boiling range 60—90 °C)/ethyl acetate(60:40, volume ratio) as eluent to afford KDP as a white powder. m. p. 92—94 °C(decomp.). ^{1}H NMR(400 MHz, $CDCl_3$), δ: 0.88(t, 6H, J=0.8 Hz, $2CH_3$), 1.25(s, 48H, $24CH_2$); 1.62—1.77(m, 4H, $2CH_2$), 2.40(t, 2H, J=7.2 Hz, CH_2), 2.59(t, 2H, J=7.2 Hz, CH_2), 4.92(s, 2H, CH_2), 6.49(s, 1H, CH), 7.87(d, 1H, CH). ^{13}C NMR (100 MHz, $CDCl_3$), δ: 183.3(CO), 178.8(C), 173.1(CO), 165.6 (C), 133.6(CH), 122.2(C), 98.7(CH), 71.3(CH_2), 20.8—35.1 ($28CH_2$), 14.8($2CH_3$). ESI-MS, *m*/*z*: 641.4736$[M+Na]^+$; found: 641.4761[20].

3 Results and Discussion

3.1 Enzyme Group Screening

The first step in this study is to find the most suitable double-enzyme combination. Four commercially available lipases(Amano PS, Novozym 435 and Lipozyme TL IM, Lipozyme RM IM) were used to group various double-enzyme combinations: Amano PS-Novozym 435, Amano PS-Lipozyme TL IM, Novozym 435-Lipozyme TL IM, Amano PS-Lipozyme RM IM, Novozym 435-Lipozyme RM IM and Lipozyme TL IM-Lipozyme RM IM. The effects of the six enzyme combinations were investigated for their ability to produce KDP ester by esterification of kojic acid with palmitic acid and the results are presented in Fig.1. In these reactions, the temperature, catalyst concentration, reaction time, mass ratio of two lipases, molar ratio of substrates, stirring speed, kojic acid concentration were kept at 55 °C, 5% of the total mass of the substrate, 400 min, 1:1(lipase 1 to lipase 2), 1:2(kojic acid to palmitic acid), 500 r/min and 100 mmol/L, respectively, using acetone as solvent. The initial reaction rates were similar for all the enzyme combinations. The KDP yield increased sharply after

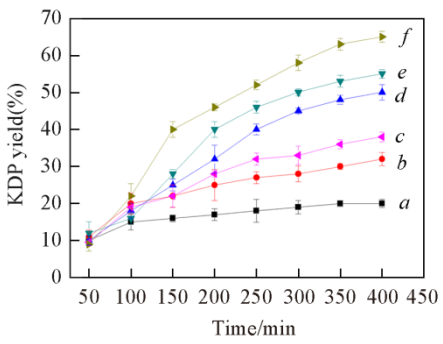


Fig.1 Effect of different enzyme groups on KDP yield

a. Lipozyme TLIM-Lipozyme RMIM; *b*. Novozym 435-Lipozyme RMIM; *c*. Novozym 435-Lipozyme TLIM; *d*. Amano PS-Lipozyme RMIM; *e*. Amano PS-Lipozyme TLIM; *f*. Amano PS-Novozym 435.

the first 100 min of reaction. The highest KDP yield(65%) was obtained with the combination of Amano PS-Novozym 435 after 400 min of reaction. The lowest KDP yield of only 20% was obtained with the combination of Lipozyme TL IM-Lipozyme RM IM. The combination of Amano PS-Novozym 435(APN) was then selected to optimize reaction conditions because of its high catalytic efficiency.

The reason why di-esterification of kojic acid could be realized more easily with double lipases than single lipase system is possibly that there is synergistic effect between the two lipases, which can improve the di-esterification efficiency. Zhang *et al*.[21] had investigated the effect of adding time of dextranase to the dextransucrase system to reveal the synergistic process of dextransucrase and dextranase. The research established the relationship between the synergistic catalysis of the double enzymatic system and realized the synthesis of oligodextrans with different molecular weights.

3.2 Effect of Different Organic Solvents

The solubility of the reaction substrates varies in different solvents, which will have effect on the reaction speed. The effect of solvent type on the esterification was investigated. In these reactions, the temperature, catalyst concentration, reaction time, mass ratio of two lipases, substrate molar ratio, stirring speed, kojic acid concentration were kept at 55 °C, 5% of the total mass of the substrate, 400 min, 1:1(Novozyme 435 to Amano PS), 1:2(kojic acid to palmitic acid), 500 r/min and 100 mmol/L, respectively. As shown in Table 1, the highest yield of KDP was about 65% when the reaction proceeded in acetone. However, the yields were not satisfactory when the reaction was performed in other organic solvents. This is because that the hydrophilicity of kojic acid and the hydrophobicity of palmitic acid make them not reach high solubility in a single polar solvent or nonpolar solvent. Acetone is a mid-polar solvent, both kojic acid and palmitic acid have relatively good solubility in it. This will increase the speed and efficiency of esterification reaction. Therefore, acetone was used as solvent for the next optimization of reaction conditions.

Table 1 Effect of organic solvents on the lipase-catalyzed esterification of kojic acid with palmitic acid

Solvent	Yield of KDP(%)
Acetonitrile	45.6
Chloroform	18.3
Diethyl ether	12.7
Cyclohexanol	30.2
Acetone	65.8
Toluene	22.4
t-Butyl alcohol	36.5
Ethyl acetate	28.3
Dioxane	17.0

3.3 Effect of Enzyme Proportion

The double-enzyme system includes two kinds of different enzymes, which differs in catalysis efficiency and regioselectivity. There should be an optimal proportion between the two enzymes. The effect of proportion of enzymes in the two-enzyme combination on the esterification has been investigated. In these reactions, the temperature, catalyst concentration, reaction time, substrate molar ratio, stirring speed, kojic acid concentration were kept at 55 °C, 5% of the total mass of the substrate, 400 min, 1:2(kojic acid to palmitic acid), 500 r/min, 100 mmol/L, respectively, and the results are depicted in Fig.2, KDP yield increased when the mass ratio of enzymes Amano PS to Novozyme 435 changed from 1:0.5 to 1:1.5, then decreased slowly as the Amano PS proportion increased further. Therefore, the optimal enzyme proportion will be about 1:1.5 (Novozyme 435 : Amano PS).

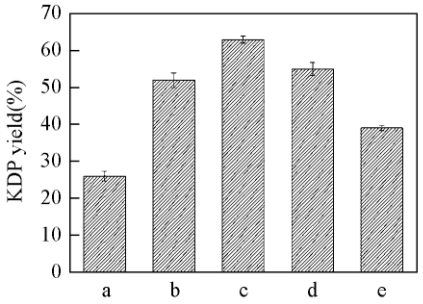


Fig.2 Effect of enzyme proportion on KDP yield

Mass ratio of Novozyme 435 to Amano PS: a. 1:0.5; b. 1:1.0; c. 1:1.5; d. 1:2.0; e 1:2.5.

3.4 Effect of Stirring Speed

A preliminary investigation has been completed to study the effect of stirring speed on this process. In these reactions, the temperature, catalyst concentration, reaction time, mass ratio of two lipases, substrate molar ratio, kojic acid concentration were kept at 55 °C, 5% of the total weight of the substrate, 400 min, 1:1(Novozym 435 to Amano PS), 1:2(kojic acid to palmitic acid), 100 mmol/L, respectively, with different stirring speeds. As can be seen in Fig.3, the reaction speed increased rapidly with increasing the stirring speed up to 500 r/min, implying that mass transfer was the rate-limiting step[18]. However, with further increasing the stirring speed over 500 r/min, the initial rate decreased because of observable aggregation of enzyme. Therefore, the stirring speed was fixed at 500 r/min.

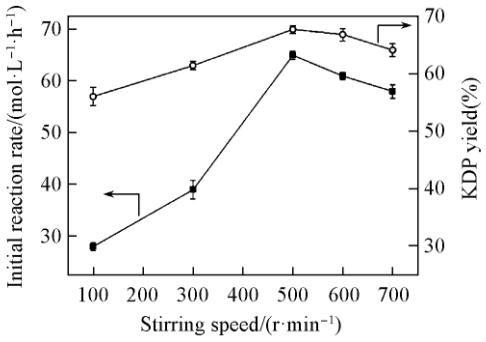


Fig.3 Effect of stirring speed on the reaction initial reaction rate and KDP yield

3.5 Effect of Molar Ratio of Substrates

The effect of molar ratio of substrates was investigated. In these reactions, the temperature, catalyst concentration, reaction time, mass ratio of two lipases, stirring speed, kojic acid

concentration were kept at 55 °C, 5% of the total mass of the substrate, 400 min, 1:1(Novozym 435 to Amano PS), 500 r/min, 100 mmol/L, respectively. Fig.4 presents the KDP yield as a function of reaction time at different molar ratios of kojic acid to palmitic acid(1:2, 1:3.5 and 1:5.5). Slight increasing was observed in KDP yield when the molar ratio increased from 1:2 to 1:3.5(excess amount of palmitic acid). This can be attributed to the chemical equilibrium shift towards the product(KDP) side, which is induced by the increasing concentration of palmitic acid. However, the yield decreased from 70% to 25% when the molar ratio of kojic acid to palmitic acid increased to 1:5.5 owing to the prohibition effect on enzymes caused by high concentration of substrates.

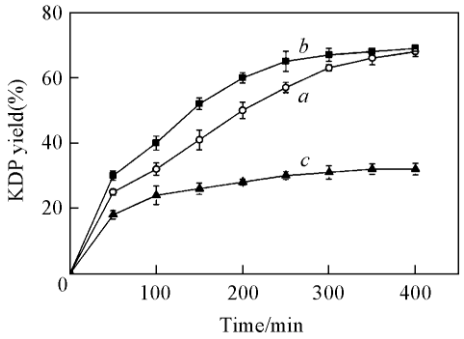


Fig.4 Effect of molar ratio of substrates on KDP yield

Molar ratio of kojic acid to palmitic acid: *a*. 1:2; *b*. 1:3.5; *c*. 1:5.5.

3.6 Effect of Reaction Temperature

The effect of temperature on the esterification was evaluated. In these reactions, the catalyst concentration, the reaction time, mass ratio of two lipases, the substrate molar ratio, stirring speed and kojic acid concentration were held constant at 5% of the total mass of the substrate, 400 min, 1:1(Novozym 435 to Amano PS), 1:2(kojic acid to palmitic acid), 500 r/min, 100 mmol/L, respectively. The range of temperature was set from 40 °C to 55 °C. Fig.5 shows that the KDP yield increased with the rise of temperature. At low temperature(40 °C), the maximum yield was less than 22%, whereas it increased to more than 80% at 50 °C. Rise of temperature could improve molecular contact between reactants, and enzyme particles and the substrates, which could speed up the reaction rate. The KDP yield reduced significantly when the temperature further increased from 50 °C to 55 °C. The possible reason was that enzyme became unstable and suffered from activity lose at higher temperature.

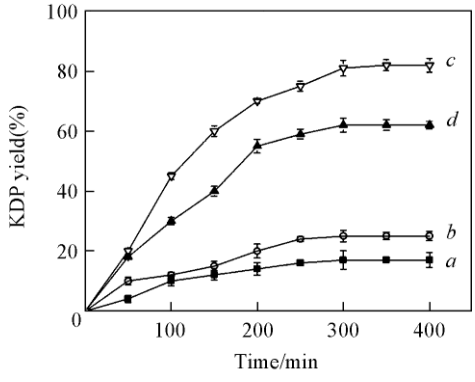


Fig.5 Effect of reaction temperature on KDP yield

Temperature/°C: *a*. 40; *b*. 45; *c*. 50; *d*. 55.

3.7 Effect of Catalyst Concentration

The effect of the catalyst concentration(mass fraction) on KDP yield was tested at fixed temperature(50 °C), time (400 min), mass ratio of Novozym 435 to Amano PS(1:1), substrate molar ratio of kojic acid to palmitic acid(1:2), stirring speed(500 r/min), kojic acid concentration(100 mmol/L). The results are showed in Fig.6. The yield of KDP increased linearly with the increase of enzyme concentration from 1% to 5%. However, it decreased obviously when the enzyme concentration increased from 5% to 6%, the possible reason was that the high concentration of enzyme could lead to a self-inhibition.

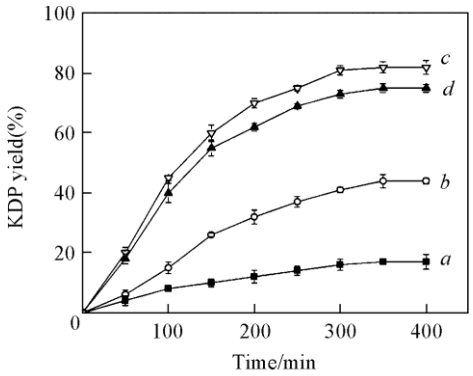


Fig.6 Effect of catalyst concentration on KDP yield

Concentration of catalyst(%, mass fraction): *a*. 1; *b*. 3; *c*. 5; *d*. 6.

4 Conclusions

In this study, C5 and C7 hydroxyl groups were esterified at the same time using the double-lipase method. Firstly, there is synergistic effect between two kinds of lipases, which has been demonstrated on the synthesis of oligodextrans[21]. Secondly, KDP was synthesized in two steps, which included the synthesis of single kojic palmitate by the esterification of kojic C5 hydroxyl group(first step) and the synthesis of KDP by the esterification of C7 hydroxyl group in single kojic palmitate molecule(second step). In the first step, double-lipase improved the yield of single kojic palmitate, which was the reactant of the second step. This would increase the reactant concentration for the second step. Finally, amphiphilic solvent was used as a co-solvent of kojic acid and palmitic acid, which could improve the chance for the contacting of reactant molecules.

The above results suggest that double-lipase could be effectively used for kojic dipalmitate production. In this study, different double-lipase groups were evaluated for their catalytic activity in the reaction, and the combination of Amano PS-Novozym 435 has the best efficiency. The yield could reach 85% uner optimal reaction conditions.

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