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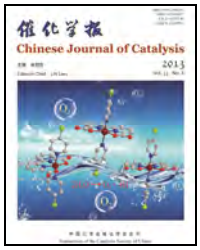

Article

Synthesis of vitamin E succinate by interfacial activated *Candida rugosa* lipase encapsulated in sol-gel materials

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ABSTRACT

Vitamin E succinate was synthesized by interfacial activated *Candida rugosa* lipase (CRL) encapsulated in sol-gel materials. The effects of various immobilization parameters were investigated. The optimum conditions were found to consist of *n*-propyltrimethoxysilane/tetraethoxysilane molar ratio = 1/1, water/silane molar ratio (*R* value) = 20, lipase loading = 0.5 mg/ml sol, and PEG400 loading = 12 μl/ml sol. Compared with free enzymes, sol-gel encapsulated enzymes kept its activity of 70.58% and achieved 2.6-fold increased stability after 18 h incubation in phosphate buffer (0.025 mol/L, pH = 7.0) at 50 °C. Based on the interfacial characteristics of CRL, five kinds of surfactants were used for activation. The results demonstrate that olive oil was the most effective in activating CRL. The esterification activity of vitamin E succinate synthesis by interfacial activated CRL encapsulated in sol-gel materials reached up to 6.7-fold and 1.43-fold that of free enzyme and non-interfacial activated enzyme, respectively.

1. Introduction

Immobilized enzymes have been used in a wide number of applications due to advantages of high stability, separation, recyclability, easy transport and storage, and continuous and automatic production [1,2]. Entrapping the enzyme in sol-gel formed by organic silane precursors further enhances its thermal stability and resistance to organic solvents, high enzymic activity, and maintains the advanced structure of the enzyme. This is an important immobilization method [3,4]. Nguyen et al. [5] reported that creatine kinase entrapped in sol-gel was obtained using tetramethyl orthosilicate as silane precursors. The results demonstrate that the thermal stability of the immobilized enzyme was greatly improved. At 60 °C the immobilized enzyme retained 50% of its maximum activity after 5 h incubation. Noureddini et al. [6] reported that lipase AY was entrapped in sol-gel formed using isobutyltrimethoxysilane and tetramethyl orthosilicate as silane precursors. The immobilized lipase was stable up to 70 °C, whereas for the free enzyme, a moderate to severe loss of activity was observed beyond 40 °C. The immobilized lipase also retained more than 95% of its initial activity after twelve reactions.

The thermal stability of the enzyme could be enhanced by the sol-gel encapsulating immobilization method. However, the catalytic efficiency of the entrapped enzyme was also low, mainly because entrapped enzyme did not have a suitable conformation to form the product. Most lipases have a lid-like structure. The substrates enter the catalytic center of lipases and the catalytic behavior is fulfilled after the "lid" of the enzyme opens. Maruyama et al. [7] demonstrated that the lid of

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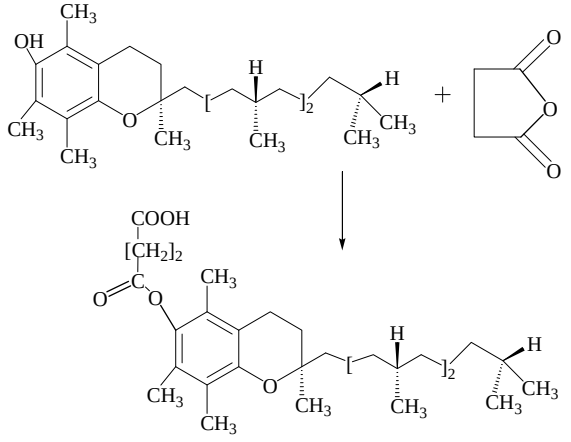
most enzymes was closed in the water phase but would open at the oil-water interface. Interface activation was not available for the organic solvents, as they cause the lid of the lipase to close and results in low catalytic efficiency. If the lid of the lipase could be opened before catalytic behavior, the catalytic efficiency could be greatly improved. Generally speaking, the lipase lid could be opened by interfacial activation formed at the oil-water phase. Foresti et al. [8] reported that *Candida rugosa* lipase (CRL) adsorbed on polypropylene powder was subjected to interfacial activation in order to enhance ethyl oleate synthesis activity in solvent-free. The esterification activity was increased by up to 29% compared with non-interfacial activated enzyme. Yilmaz [9] employed surfactants such as lecithin, Tween 20, and olive oil to activate porcine pancreatic lipase and *Pseudomonas fluorescens* liapse. The results show that the interesterification activities of both porcine pancreatic lipase and *Pseudomonas fluorescens* lipase were quadrupled by lecithin.

Vitamin E succinate is one of the important vitamin E derivatives. It has remarkable effects against all kinds of tumors, including colon and breast tumors. In particular, it has no influence on the proliferation of normal cells, making it a potential anti-cancer drug [10,11]. Recently, to ensure environmentally friendly processing, synthesis by enzyme-catalyzed reactions instead of traditional chemical catalysts has become of commercial interest [12,13]. Biocatalysts have been widely used in many kinds of reactions. For example, the Michael addition reaction, Markovnikov addition reaction, and aldol condensation use biocatalysts [14,15]. Torres et al. [16] described the enzymatic acylation of vitamin E with vinyl acetate in 2-methyl-2-butanol for the first time, in which Novozym 435 was chosen as the catalyst, reaching a yield of about 65% after 18 days. Yin et al. [17] described the synthesis of vitamin E succinate using a chemically modified Novozym 435 in *tert*-butanol and DMSO (v/v = 2:3). The yield of vitamin E succinate reached 94.4% after 48 h, while the yield was only 13.13% with unmodified Novozym 435. In this study, sol-gel encapsulating immobilization and interfacial activation were employed to improve the esterification activity of CRL in the synthesis of vitamin E succinate (Scheme 1). This research also explored the influence of different kinds of enzymes and organic solvents, as well as the operating conditions on the synthesis of vitamin E succinate [18].



Scheme 1. Synthesis pathway of vitamin E succinate by interfacial activated *Candida rugosa* lipase (CRL) encapsulated in sol-gel materials.

2. Experimental

2.1. Preparation for CRL encapsulated in sol-gel materials

A certain amount of CRL (Sigma-Aldrich) was first dissolved in phosphate buffer (0.025 mol/L, pH = 7.0) and centrifuged (6000 r/min, 4 °C, 10 min) to remove any insoluble material. The supernatant was obtained. A mixture of *n*-propyltrimethoxysilane (PTMS, 97%, Aladdin-reagent)/tetraethoxysilane (TEOS, AR, Aladdin-reagent, 12 mmol), 1.5 ml deionized water, HCl solution (1 mol/L, 90 μl), and a certain amount of polyethylene glycol 400 (PEG400, Sinopharm Chemical Reagent Co. Ltd.) were intensively agitated at 0 °C for 30 min. The supernatant was added to the above hydrolyzed silane solution. The mixture was vigorously agitated for 10 min and then at 100 r/min for another 1 h. The agitated mixture was aged at 4 °C for 24 h, then dried in air at 30 °C for 3 d. Finally, the generated gel was crushed and the immobilized enzyme was obtained.

2.2 Preparation for interfacial activated CRL encapsulated in sol-gel materials

A certain amount of lipase powder was dissolved in phosphate buffer (0.025 mol/L, pH = 7.0) and centrifuged (6000 r/min, 4 °C, 10 min) to remove any insolubles. The supernatant was obtained. A certain amount of olive oil, triacetin, Tween 20, lecithin, and *n*-octyl glucoside (*n*-OG, Sinopharm Chemical Reagent Co. Ltd.) was added into the supernatant. Sol-gel immobilization was performed as described in Section 2.1. Interfacial activated CRL encapsulated in sol-gel material was dried and crushed. *n*-OG and Tween 20 were washed with benzene/ethanol (90/10). Olive oil, triacetin, and lecithin were washed with *n*-hexane. The interfacial activated CRL encapsulated in sol-gel materials was then dried in air.

2.3. Immobilization efficiency assay

The dried sol-gel was washed by a phosphate buffer and the washed buffer collected. Protein was determined according to Bradford's method using bovine serum albumin (Aladdin-reagent) as a standard [19]. The immobilization efficiency (IY) was calculated from the formula: $IY = (C_i - C_f/C_i) \times 100\%$, where C_i and C_f mean the concentration of the initial and final enzyme protein concentrations in the immobilization medium (mg/ml).

2.4. Specific surface area and pore diameter assays

N_2 adsorption-desorption assays of silane particles were conducted on a Micromeritics ASAP 2020 analyzer. All the samples were degassed at 80 °C for 12 h before measurement. Specific surface area was determined by BET model, and the

pore diameter was determined by BJH method [18].

2.5. Hydrolysis activity assays

The hydrolysis activity of enzyme was determined by olive oil hydrolysis. A mixture of 8.5 g of gum arabic powder (Sinopharm Chemical Reagent Co. Ltd), 100 ml of deionized water, and 100 ml of olive oil (AR, Sinopharm Chemical Reagent Co. Ltd) was stirred overnight at room temperature to prepare the olive oil emulsion. The enzyme solution consisted of 10 ml phosphate buffer (0.025 mol/L, pH = 7.0), 10 ml olive oil emulsion and a certain amount of enzyme. The mixture was continuously titrated with 0.025 mol/L NaOH to maintain the pH at 7.0. The hydrolysis temperature was strictly controlled at 35 °C. The volume of NaOH consumed in 10 min was recorded. Hydrolysis activity (U) was determined by calculating the consumption of NaOH and was defined as micromoles per minute per gram of protein.

2.6. Thermal stability assays

A certain amount of enzyme was dissolved in phosphate buffer (0.025 mol/L, pH = 7.0) at 30–60 °C for 18 h. The enzyme solution was then cooled and added into the olive oil system. The hydrolysis activity was measured as 2.5.

2.7. Esterification activity assays

Enzymatic esterification was typically performed in a reaction mixture consisting of 1 mmol vitamin E (AR, Sinopharm Chemical Reagent Co. Ltd), 5 mmol succinic anhydride (AR, Sinopharm Chemical Reagent Co. Ltd), a certain amount of free enzyme, enzyme encapsulated in sol-gel material, or interfacial activated enzyme encapsulated in sol-gel material, and 5 ml DMSO (AR, Sinopharm Chemical Reagent Co. Ltd). The reaction was performed in tightly closed 25 ml conical flasks, in a water bath of 55 °C, agitated with mechanical stirring at 150 r/min and a reaction time of 18 h. Quantitative analysis of the reactants and products were conducted by HPLC system from Shimadzu, Japan. A reversed-phase column (Sepax BR-C18, 250 mm × 4 mm, 5 μm) was used and the reactants and products detected by SPD-20AVP UV-Vis detector at 285 nm. A mixture of methanol/acetic acid, 50/0.3 (v/v), was used as an eluent at 36 °C with a flow rate of 1 ml/min. Esterification activity was determined by calculating the transformation of the substrate (vitamin E) to ester, and was defined as micromoles per hour per gram of protein.

3. Results and discussion

3.1. Effects of immobilization conditions on the immobilized CRL

3.1.1. Effect of molar ratio of silane precursors

The molar ratio of the hydrophobic and hydrophilic silane precursors determines the hydrophobicity of the gel. When the ratio is higher, the hydrophobicity of the growing gel results in microscopic phase separation, during which the aqueous phase solution might be squeezed out of the gel phase. Hence, when the phase separation and the gelation proceeded at a certain balanced rate, some lipase molecules could be aptly trapped near the surface of the resulting gel [20]. As lipases are interface active enzymes, a suitable surface hydrophobicity of the gel might be helpful to maintain favorable molecular conformation and hence enhance activity [3]. So an optimum molar ratio of the hydrophobic and hydrophilic silane precursors is desirable. The effect of the molar ratios of hydrophobic PTMS and hydrophilic TEOS on the specific activity and immobilized protein ratio of the immobilized enzymes were investigated and the results are shown in Fig. 1. When the amount of PTMS decreased in the precursor mixture, the immobilization protein ratio fell, while the specific activity first enhanced and then decreased, achieving a maximum value at the molar ratio of 1/1. Therefore, 1/1 was selected as the optimum silane precursor molar ratio for further experiments.

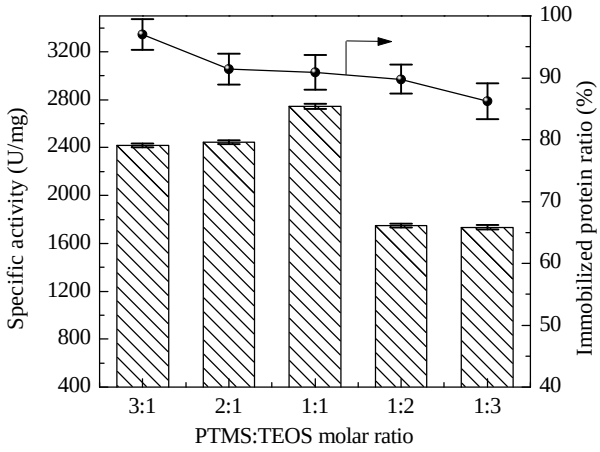


Fig. 1. Effect of the PTMS to TEOS molar ratio on the specific activity and immobilized protein ratio of immobilized enzymes.

3.1.2. Effect of lipase loading

Lipase loading directly influences the support loading and indirectly influences the specific activity of immobilized enzymes. Enzyme denaturation by the hydrophobic gel material with low lipase loading might explain the initial activity increase [21]. Intraparticle diffusion limitations and/or enzyme aggregation may be responsible for decreased activity at high enzyme loading. When the PTMS/TEOS molar ratio was 1:1, the water/silane precursor molar ratio (*R* value) was 20, and the amount of PEG was 80 μl/ml sol, the effect of varied lipase loading on immobilization was investigated and the results are shown in Fig. 2. The specific activity and immobilized protein ratio increased with increasing enzyme loading when the lipase loading was less than 0.5 mg/ml. The specific activity and immobilized protein ratio decreased with increasing enzyme loading when the lipase loading was above 0.5 mg/ml. When the lipase loading was 0.5 mg/ml, the highest values of specific activity and immobilized protein ratio were achieved.

3.1.3. Effect of the molar ratio of water to silane precursor

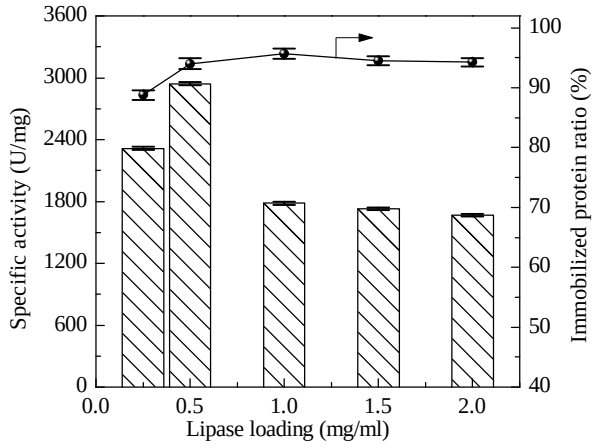


Fig. 2. Effect of lipase loading on specific activity and immobilized protein ratio of immobilized enzymes.

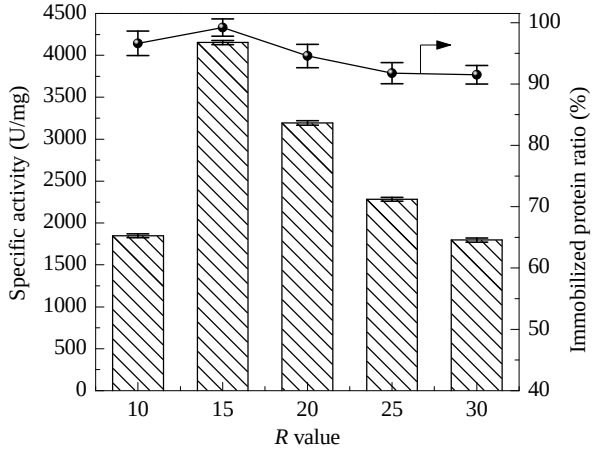


Fig. 3. Effect of the R values on specific activity and immobilized protein ratio of immobilized enzymes.

The molar ratio of total water to silane precursor was usually reflected by the *R* value in the gel-forming reaction. As shown in Fig. 3, the specific activity and immobilized protein ratio were low when the *R* values < 15. The specific activity and immobilized protein ratio decreased when the *R* values > 15. Both the specific activity and immobilized protein ratio achieved the highest values when the *R* value = 15. Lower *R* values might result in enzyme aggregation, which leads to low activity. Alcohol condensation was also likely to occur during network forming and might damage the enzymes. However, at higher *R* values, more lipase molecules remained in the aqueous supernatant, so a lower immobilized protein ratio resulted [22].

3.1.4. Effect of PEG400 amount

PEG400 adsorbed onto the surface of the gel before the enzyme, which weakened interactions between the enzyme and gel. PEG400 was able to maintain enzyme activities because of their satisfactory biocompatibility. The amount of PEG400 had a significant influence on the gel structure [23,24].

As shown in Fig. 4, when the amount of PEG400 increased, the specific activity was first raised and then declined, with a

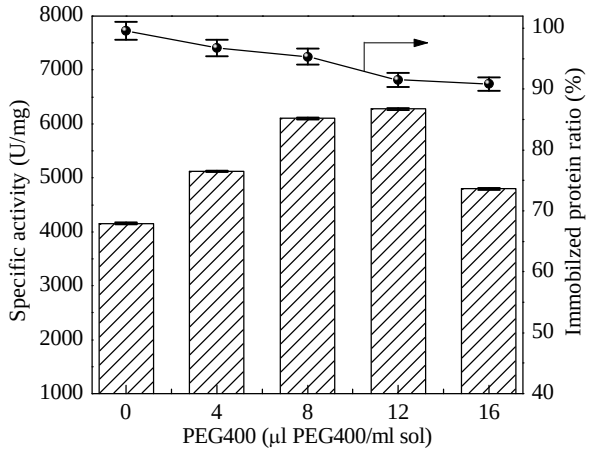


Fig. 4. Effect of PEG on the specific activity and immobilized protein ratio of immobilized enzyme.

maximum value (6279.12 U/mg) at the point of 12 μl/ml. The specific activity improved by 3.6 times that of free enzymes. The immobilized protein ratio reached 95.3%. The optimum immobilized conditions of PTMS/TEOS = 1/1 (mol/mol), water/silane molar ratio (*R* value) = 20, lipase loading = 0.5 mg/ml sol, and PEG400 loading = 12 μl/ml sol were found by exploring the immobilized conditions.

3.1.5. Specific surface area and pore diameter of the gels

The specific surface area and pore diameter of the gels were also determined by N_2 adsorption-desorption assays. As shown in Table 1, compared with the blank gels, the specific surface area of the immobilized enzymes increased, while the pore diameter of the immobilized enzymes decreased. Enzyme molecules were encapsulated in the sol-gel material in the gel-forming reaction. The structure of the gels was loose and porous, which resulted in high specific surface area. The pore diameter of the gels decreased because enzyme was encapsulated in sol-gel materials. These changes demonstrated that the enzyme was successfully encapsulated in the sol-gel materials. The specific surface area of the immobilized enzymes was also bigger than that of the blank gels, which favors movement of the substrates and products coming in and out of the enzyme catalytic active center. This results in high enzyme catalytic efficiency [25,26].

3.2. Enzymatic properties

3.2.1. Thermal stability

Thermal stability is an important character of enzymes. Nguyen et al. [5] reported that enzymes by sol-gel encapsulation obtain higher stability. The thermal stability of the lipase encapsulated by PTMS/TEOS was investigated at the immobi-

Table 1
BET surface area and average pore width.

Sample	BET surface area (m^2/g)	Average pore width (nm)
Blank	43.23	4.23
Immobilized enzyme	54.71	3.26

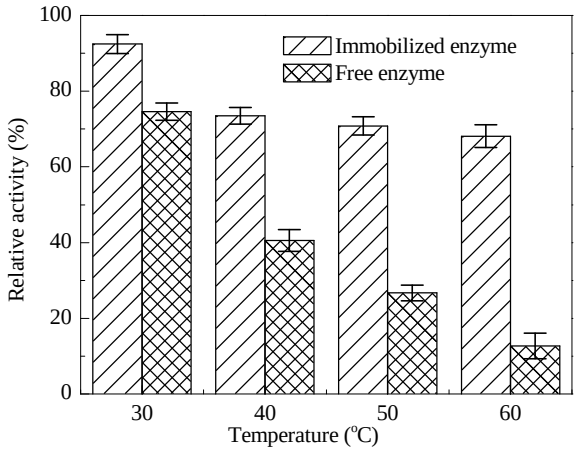


Fig. 5. Thermal stability of free and immobilized enzyme.

lization conditions described above. It was shown in Fig. 5 that the improved thermal resistance was achieved after immobilization. Especially at high temperatures of 50 and 60 °C, the immobilized enzyme kept 70.58% and 68.11% of its enzymatic activity, while the free enzyme kept 26.72% and 12.68%, respectively. The enhanced stability of the sol-gel encapsulated enzymes might be attributed to the global movement (e.g. unfolding, rotating) being restricted by the rigid polymer [24].

3.2.2. Comparison of esterification activity of CRL by interfacial activation with different surfactants

The difference between lipase and other esterase lies in the performance of the lipase-catalytic behavior at the oil-water interface, which was determined by the unique "lid" structure. The crystallography of the lipase demonstrated that there was a helicoid lid covering the catalytic triad of the lipase, which separated the catalytic center from the surface of lipase. There was an electrophilic area (oxygen anion hole) formed by the hydrophobic and hydrophilic groups under the lid [25]. The lid was open and the catalytic center was exposed to the water-organic interface. Therefore, the substrates could easily combine in the catalytic center. From the data presented in Table 2**,** it was demonstrated that the esterification activity of the interfacial activated immobilized lipase improved by varying degrees compared with free and immobilized lipase. Among the surfactants tested, the best surfactant was olive oil and the esterification activity of the interfacial activated lipase encapsulated in sol-gel materials was 7.59×10^4 U. The esterification activity of the free enzyme was 1.13×10^4 U. The esterification activity of vitamin E succinate synthesis by interfacial activated CRL encapsulated in sol-gel material reached up to 6.7 times and 1.43 times that of free enzymes and non-interfacial activated immobilized enzymes, respectively. This was because the hydrophobicity of olive oil was the strongest and the two-phase system was well formed. The lipase lid could effectively open and the esterification activity of vitamin E succinate synthesis by CRL was greatly improved [8,27,28]. Compared with previous reports, the esterification activity of vitamin E succinate synthesis by interfacial activated CRL encapsulated in sol-gel materials was obviously improved more than Novozym 435 [16,17].

Table 2
Esterification activity of encapsulated CRL by interfacial activation with different surfactants for the synthesis of vitamin E succinate.

Surfactant	Esterification activity (U)	Change
Blank	5.31×10^4	1
Triacetin	5.98×10^4	1.13
Lecithin	6.25×10^4	1.17
Tween 20	6.65×10^4	1.25
n-OG	6.77×10^4	1.27
Olive oil	7.59×10^4	1.43

4. Conclusions

The effects of various immobilization conditions on the synthesis of vitamin E succinate by interfacial activated *Candida rugosa* lipase (CRL) encapsulated in sol-gel materials were investigated. These conditions involved the molar ratio of silane precursors, lipase loading, *R* value, and amount of PEG400. Optimum conditions were found where *n*-propyltrimethoxysilane/tetraethoxysilane = 1/1 (mol/mol), water/silane molar ratio = 20, lipase loading = 0.5 mg/ml sol, and PEG400 loading = 12 μl/ml sol, the specific activity was 6279.12 U/mg protein and the immobilized protein ratio was 95.3%. The thermal stability and hydrolysis activity were also obviously improved. Lecithin, *n*-OG, olive oil, triacetin, and Tween 20 were used to activate CRL based on the interfacial characteristics of CRL. Compared with non-interfacial activated enzymes, the esterification activities were improved by varying degrees. The results demonstrate that olive oil has the greatest effect on CRL. The esterification activity of vitamin E succinate synthesis by interfacial activated CRL encapsulated in sol-gel materials reached up to 6.7 times and 1.43 times that of free enzymes and non-interfacial activated immobilized enzymes, respectively. Therefore, the sol-gel encapsulating immobilized method and interfacial activation could effectively reform and improve the catalytic ability of the enzyme.

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Graphical Abstract

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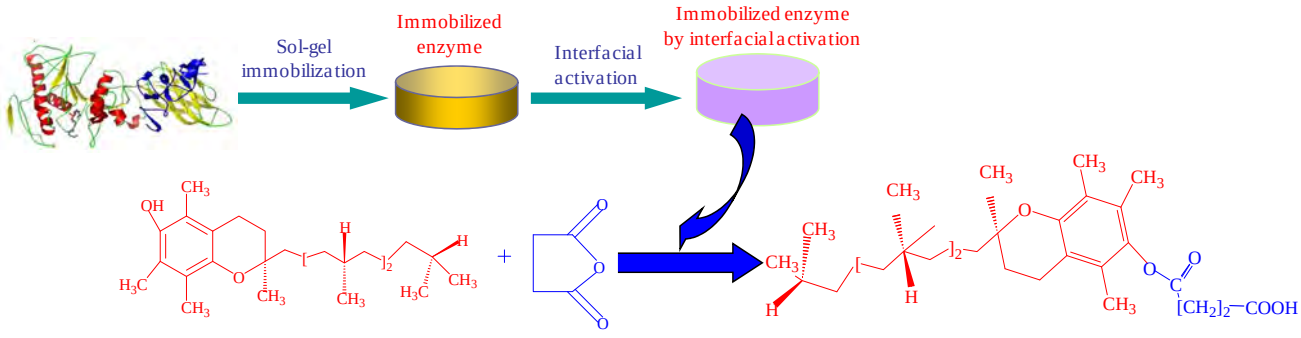

Interfacial activated *Candida rugosa* lipase encapsulated in sol-gel materials was prepared and used to synthesize vitamin E succinate.

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界面活化的溶胶凝胶包埋*Candida rugosa*脂肪酶催化合成维生素E琥珀酸酯

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摘要: 采用界面活化的溶胶凝胶包埋*Candida rugosa*脂肪酶(CRL)催化合成了维生素E琥珀酸酯. 考察了影响溶胶凝胶包埋固定化CRL的因素, 获得的最佳固定化条件为: 丙基三甲氧基硅烷/正硅酸四乙酯摩尔比为1/1, 水与硅烷前体摩尔比为15, 酶的添加量为0.5 mg/ml, PEG400的添加量为12 μl/ml溶胶. 溶胶凝胶包埋的CRL在50 ºC, 18 h后其活性仍然保持了70.58%, 是游离酶的2.6倍, 且稳定性得到了明显的改善. 基于CRL的界面特性, 采用五种表面活性剂对其进行界面活化. 结果表明, 采用橄榄油活化的溶胶凝胶包埋的CRL合成维生素E琥珀酸酯的酯化活力最高, 相比原酶和未界面活化的溶胶凝胶包埋酶分别提高了6.7和1.43倍.

关键词: 溶胶凝胶; 界面活化; *Candida rugosa*脂肪酶; 维生素E琥珀酸酯

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1. 前言

固定化酶具有稳定性好、易从反应系统中分离、能重复使用、便于运输和贮存、有利于生产的连续化和自动化等优点, 因而具有广阔的应用前景[1,2]. 以有机硅烷为前体的溶胶凝胶包埋固定化可有效地提高酶的热稳定性和耐有机溶剂性、酶活回收率高、能维持酶的高级结构等特点, 成为当前酶固定化重要方法之一[3,4]. Nguyen等[5]以硅酸甲酯为前体对肌酸激酶进行溶胶凝胶包埋固定化, 发现所得固定化酶的热稳定性明显提高, 溶胶凝胶包埋的酶在60 ºC使用5 h仍能保持50%的活性, 而游离酶反应1 h就失去了50%的活性. Noureddini等[6]以异丁基三甲氧基硅烷和硅酸甲酯为前体, 采用溶胶凝胶包埋脂肪酶AY. 结果表明, 40 h后游离酶在40 ºC以上会失去大部分活性, 而固定化酶在70 ºC时依然比较稳定, 重复使用12次后依然能保持95%的活性.

虽然利用溶胶-凝胶包埋固定化法能有效提高酶的稳定性, 但是经过包埋的酶依然存在催化效率低的问题. 这主要是因为包埋酶的构象并非是形成产物的最佳构象. 大多数脂肪酶都有一个“盖子”结构, 只有当该盖子结构打开之后, 底物才能进入其催化活性中心完成催化过程. Maruyama等[7]已经证明, 大多数脂肪酶在水相中盖子结构处于关闭状态, 而在油水的两相界面中常常处于打开的状态. 而在有机溶剂中, 由于缺少这种“界面激活”作用, 脂肪酶的盖子结构通常是关闭的, 这是导致脂肪酶在有机溶剂中催化效率低的重要因素之一. 如果能够在催化前预先打开脂肪酶的盖子结构, 那么就能有效提高脂肪酶的催化效率. 脂肪酶的盖子结构可在油水两相体系中通过界面活化作用来打开. Foresti等[8]采用油水界面活化的聚丙烯固定的*Candida rugosa*脂肪酶(CRL)在无溶剂体系中催化油酸和乙醇合成油酸乙酯, 相比未界面活化的固定化酶, 其酯化活力提升了29%. Yilmaz[9]利用卵磷脂、吐温20、橄榄油等表面活性剂对猪胰脂肪酶和*Pseudomonas fluorescens*脂肪酶进行界面活化, 在正己烷中催化辛酸乙酯和三油酸甘油酯进行酯交换反应, 研究表明利用卵磷脂活化的猪胰脂肪酶和*Pseudomonas fluorescens*脂肪酶酯交换活力都升高了4倍左右.

维生素E琥珀酸酯是一种重要的维生素E衍生物, 除具有维生素E的功能外, 对各类肿瘤及恶性肿瘤均有显著的作用, 且不影响正常细胞的增殖, 是一种潜在的抗癌药物[10,11]. 随着近年来人们对绿色合成工艺的重视, 利用生物催化剂来代替传统的化学催化剂已成为当前研究的热点[12,13]. 生物催化剂在Michael加成、Markovnikov加成及羟醛缩合等多种催化反应中有着重要的应用[14,15]. Torres等[16]首次报道了利用Novozym 435在叔戊醇中催化合成维生素E的酯化反应, 反应18 d后底物转化率仅为65%. Yin等[17]在叔戊醇和DMSO的混合溶液(v/v = 2:3)中进行酸酐修饰的Novozym 435催化合成维生素E琥珀酸酯实验, 反应18 h后底物转化率为94.4%, 然而未修饰的Novozym 435仅为13.13%. 本课题组研究了影响合成维生素E琥珀酸酯(图式1)的脂肪酶种类、有机溶剂种类与工艺条件[18], 为进一步提高维生素E琥珀酸酯合成效率, 本文拟通过溶胶凝胶包埋固定化和界面活化的方法对CRL进行改造, 以提高原酶的酯化活力.

2. 实验部分

2.1. 溶胶凝胶固定化CRL的制备

将一定质量的CRL (购自Sigma-Aldrich公司)溶解到磷酸缓冲溶液中(0.025 mol/L, pH = 7.0), 离心(转速6000 r/min, 4 ºC, 10 min), 取上清液备用. 按一定摩尔比的丙基三甲氧硅烷(PTMS, 97%, Aladdin-reagent)和正硅酸四乙酯(TEOS, AR, Aladdin-reagent) 12 mmol高速搅拌混合均匀, 依次加入1.5 ml去离子水、盐酸(1 mol/L, 90 μl)和一定量的聚乙二醇400 (PEG400)搅拌30 min后得到均匀的硅烷前体. 将上清液加入到硅烷前体中, 搅拌10 min后转为低速继续搅拌1 h, 再放入4 ºC陈化24 h. 陈化结束后, 在30 ºC下真空干燥3 d, 待产物干燥后磨成颗粒即为固定化酶.

2.2. 界面活化的溶胶凝胶固定化CRL的制备

取一定量的CRL加入到磷酸缓冲液(0.025 mol/L, pH = 7.0)中, 然后离心(6000 r/min, 4 ºC, 10 min), 取上清分别加入一定量的橄榄油、三乙酸甘油酯、吐温20、卵磷脂和*n*-OG, 搅拌30 min, 而后对其进行溶胶凝胶固定化(固定化过程如2.1节). 界面活化的溶胶凝胶固定化CRL经干燥和磨成颗粒后, 用苯/乙醇(90/10, v/v)冲洗去*n*-OG和吐温20, 用正己烷冲洗去除橄榄油、三乙酸甘油酯和卵磷脂. 自然晾干得界面活化的溶胶-凝胶固定化酶.

2.3. 固定化效率的测定

将干燥后的凝胶固体取出后, 用磷酸盐缓冲液多次清洗并收集洗涤液. 按照Bradford的方法, 以牛血清蛋白作为标准蛋白质绘制标准曲线[19]. 测定固定化后残

液的吸光值, 根据标准曲线计算蛋白浓度. 根据下式计算固定化效率IY = (C_i−C_f)/C_i×100%. 其中, C_i表示溶液中固定化前酶蛋白的初始浓度(mg/ml); C_f表示固定化过程中未被结合的酶蛋白的最终浓度(mg/ml).

2.4. 比表面积和孔径大小的测定

样品的N_2吸附-脱附曲线在Micromeritics ASAP 2020型自动吸附仪上测定. 实验前于80 °C脱气12 h, 比表面积采用BET法计算, 孔径大小由BJH方法算得[18].

2.5. 水解活性测定

酶水解活性采用橄榄油水解法测定. 将8.5 g阿拉伯树胶粉, 100 ml去离子水和100 ml橄榄油混合后于室温高速过夜搅拌得到橄榄油乳化液. 分别取10 ml橄榄油乳化液和10 ml (pH = 7.0, 0.025 mol/L)磷酸缓冲溶液于50 ml烧杯中混匀, 加入一定量的酶, 在保持pH 7.0, 反应温度为35 ºC, 反应过程产生的酸在电位滴定仪上采用0.025 mol/L的NaOH连续滴定, 并记录10 min内消耗NaOH溶液的体积. 酶活单位(U)的定义为每分钟催化橄榄油生成1 μmol脂肪酸所需的酶量. 比活力(U/g) = 固定化CRL的表观活力(U)/固定化酶中CRL的蛋白含量(g).

2.6. 热稳定性的考察

将一定量的游离酶或固定化酶加入到一定体积的磷酸盐(pH = 7.0, 0.025 mol/L)缓冲液中, 并置于30–60 ºC水浴锅中保温18 h, 然后取出, 冷却后加入到橄榄油体系中, 按照2.5节的方法测定水解活力.

2.7. 酯化活性考察

取1 mmol维生素E (AR, 购自Sigma-Aldrich公司), 5 mmol丁二酸酐(AR, 国药集团化学试剂有限公司)和一定质量的原酶、溶胶凝胶固定化酶或者界面活化的溶胶凝胶固定化酶加入到含有5 ml DMSO的25 ml具塞锥形瓶中, 在55 ºC恒温水浴振荡器中反应18 h (转速为100 r/min). 反应结束后采用配有SPD-20AVP UV-Vis检测器的岛津HPLC进行分析, 色谱柱(Sepax BR-C18, 250 mm × 4 mm, 5 μm), 温度36 ºC, 流动相甲醇/乙酸(50/0.3, v/v), 检测波长285 nm, 流速1 ml/min. 酯化活力定义为每克酶蛋白每小时催化底物(维生素E)转化为1 μmol产物维生素E琥珀酸酯的量(μmol/(h·g))

3. 结果与讨论

3.1. 固定化条件对固定化CRL的影响

3.1.1. 硅烷前体比例的影响

疏水性和亲水性的硅烷前体的比例将决定凝胶材料的疏水性. 当该比例较高时, 由于凝胶的疏水性较强, 其在聚合中容易使水“挤出”, 造成凝胶相与水相分离. 因此, 只有当两相的分离速度与凝胶聚合的速度达到平衡时, 酶分子才能被包住[20]. 由于脂肪酶是一种界面酶, 适宜的表面疏水性将有利于酶分子维持有利的催化构型[3]. 所以, 疏水性和亲水性硅烷前体的比值存在最佳值. 因此, 首先考察了疏水性的PTMS和亲水性的TEOS的比例对固定化酶的比活和蛋白负载量的影响, 结果见图1. 可以看出, 随着混合物中前体PTMS含量的降低, 蛋白结合率下降; 固定化酶的比活力先升后降, 在1:1时达到最大值. 因此, 确定将硅烷前体的比例1:1作为后续固定化的最佳条件.

3.1.2. 酶添加量的影响

酶的添加量将直接影响固定化载体的负载量, 也会间接对固定化酶的比活产生重要的影响. 酶的负载量较低时, 酶分子容易受到凝胶载体的强疏水性作用而使其有利的构象发生扭曲. 因此, 随着酶负载量的增加, 酶的比活会升高[21]. 而酶添加量较高则容易导致酶的聚集以及扩散限制等效应, 降低酶的催化效率. 图2考察了当PTMS/TEOS为1:1, 水和硅烷的摩尔比(R值)为20, PEG的量为80 μl/ml时不同的酶添加量对固定化的影响. 由图可见, 当酶的添加量小于0.5 mg/ml时, 随着酶浓度的增加, 固定化酶的比活以及固定蛋白量都在增加. 当酶的添加量大于0.5 mg/ml时, 酶的比活和固定化效率均在降低. 即当酶的添加量为0.5 mg/ml时固定化酶的酶活和固定化效率最佳.

3.1.3. 水添加量的影响

在溶胶凝胶制备过程中通常采用R值来表示添加水和硅烷前体摩尔比值. 如图3所示, 当R值<15时, 酶活和固定化效率都较低; 当R值>15时, 固定化酶的活性和固定化效率下降; 而在R = 15时, 固定化酶的比活和蛋白负载量都达到最大. 这可能是由于当R值较低时, 酶分子容易发生聚集, 造成催化效率降低. 同时, 由于前体水解和聚合过程中产生乙醇, 低的水添加量将导致乙醇浓度过高, 对酶蛋白不利. 然而当R值较高时, 酶分子则容易留在水相中, 使得酶蛋白的结合效率下降[22].

3.1.4. PEG400的添加量的影响

PEG400可先于酶吸附在凝胶表面, 从而减弱酶与凝胶间相互作用, 而且PEG400具有良好的生物相容性, 能较好地保持酶的活性. 此外, PEG400的添加量对凝胶网的结构也有较大的影响[23,24].

如图4所示, 随着PEG400量的增加, 包埋酶的比活

先升高, 12 μl/ml时达最大值为6279.12 U/mg, 比游离酶的1732.8 U/mg提高了3.6倍, 而此时蛋白固定化效率达到了95.3%. 综上所述, 固定化的最佳条件为硅烷前体摩尔比为1:1, 酶添加量0.5 mg/ml, R = 15, PEG400的添加量12 μl/ml.

3.1.5. 溶胶凝胶的比表面积和孔径

表1为比表面积和孔径大小. 可以看出, 包埋酶的溶胶凝胶颗粒的比表面积大于未包埋酶的溶胶凝胶的表面积大, 而孔径相对较小. 这是由于在凝胶形成的过程中大分子的酶分子被包埋在其中, 形成的凝胶结构比未包埋酶的疏松多孔, 因而比表面积大. 另一方面, 由于酶被包埋在孔道中, 其孔径相对未包被酶的凝胶来说会小, 这些变化说明了CRL成功地被包埋在凝胶中. 此外, 固定化酶的比表面积比空白的大, 有利于底物和产物出入酶的催化活性中心, 使得酶的催化效率较高[25,26].

3.2. 酶学性质考察

3.2.1. 热稳定性

热稳定性是酶非常重要的一种性质, Nguyen等[5]发现, 利用溶胶凝胶包埋可有效提高酶的热稳定. 图5考察了在上述最佳固定化条件下制备的固定化酶的热稳定性. 可以看出, 经过固定化后, 脂肪酶的稳定性明显改善. 特别在50和60 ºC时, 固定化酶分别能保持70.58%和68.11%的酶活, 而游离酶的酶活仅保持26.72%和12.68%. 溶胶凝胶包埋酶稳定性得到提高的原因可能在于酶分子被包被在刚性的聚合物里, 其运动(如折叠和旋转)受到限制[24].

3.2.2. 不同表面活性剂界面活化的CRL酯化活力比较

与其它酯酶不同, 脂肪酶的催化过程是在油-水界面间完成, 因此表现出特有的界面活性(interfacial activation). 这是由脂肪酶独特的盖子结构决定的, 脂肪酶结构晶体学的研究表明, 脂肪酶的活性位点上有一个螺旋型的盖子, 它的存在使酶的活性中心与分子表面被隔开, 盖子下面覆盖的是由疏水基包围的、由亲水基组成的脂肪酶亲电区(也称作氧负离子洞)[25,26]. 在由表面活性剂和水形成的两相界面中, 脂肪酶的盖子结构会被打开, 催化活性中心就暴露出来, 这样就很容易与底物相结合. 如表2所示, 相比游离酶和未界面活化的溶胶凝胶固定化酶, 经表面活性剂界面活化的酶酯化活力均有所增加, 其中效果最好的表面活性剂为橄榄油, 对应的酯化活力是未界面活化的溶胶凝胶酶的1.43倍, 高达7.59×10^4 U; 是原酶(1.13×10^4 U)的6.7倍. 这可能是由于在所选择的表面活性剂中以橄榄油的疏水性最强, 形成的两相体系效果最好, 能有效打开脂肪酶的盖子结构, 因而明显地提高了CRL合成维生素E琥珀酸酯的反应活性[8,27,28], 其酯化活性明显高于Novozym 435 [16,17].

4. 结论

对影响溶胶-凝胶包埋固定化的硅烷前体的比例, 酶、水和PEG400的添加量等因素进行了考察, 获得的最佳固定化条件为: 硅烷前体的摩尔比为1:1, 酶的添加量为0.5 mg/ml, R = 15, PEG400的添加量为12 μl/ml, 固定化效率为95.3%, 水解活力为6279.12 U/mg. 相比游离的CRL, 其热稳定性和水解活性明显提高. 利用橄榄油、三乙酸甘油酯、吐温20、卵磷脂和*n*-OG等表面活性剂对溶胶凝胶包埋的CRL进行界面活化, 发现其酯化活力明显高于原酶和未界面活化的溶胶凝胶固定化酶, 其中以橄榄油活化效果最为明显, 催化合成维生素E琥珀酸酯的酯化活力提升到原来的1.43倍, 是原酶的6.7倍. 因此, 利用溶胶-凝胶包埋法和界面活化能有效地对酶进行改造, 并定向改善酶的催化合成能力.