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Enzymatic synthesis of gallic acid esters using microencapsulated tannase: effect of organic solvents and enzyme specificity

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Abstract

Enzymatic synthesis of gallic acid esters from gallic acid and alcohols in organic solvents was studied using microencapsulated tannase from *Aspergillus niger*. Microencapsulated tannase showed higher synthetic activity than free enzyme. The water content of the system can significantly affect the equilibrium shift. In water–hexane system, the equilibrium-controlled reaction was favorable to synthesis of propyl gallate with increase of organic solvent content. The effects of various organic solvents ($\log P$: −1.3 to 6.6) on the enzymatic reactions were investigated and the highest yield was 44.3% in benzene ($\log P$: 2.0). Enzyme specificity for the alcohol portion (C_1–C_{18}) of the ester showed that the most suitable substrate was 1-propanol.
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Keywords: Tannase; Gallic acid esters; Organic solvents; Solvent hydrophobicity; Microencapsulation

1. Introduction

Biocatalysis in organic media has been the object of intensively basic and application-orientated research. From a biotechnological perspective there are many advantages of employing enzymes in organic as opposed to aqueous media [1]. Some of these include dramatically higher substrate solubility, the ability to use enzymes synthetically rather than degradatively, and the capability to modify native selectivity by simply tailoring the reaction medium rather than the enzyme itself.

Tannin acyl hydrolases (EC 3.1.1.20) are esterases able to hydrolyze the 'ester' bond (galloyl ester of an alcohol moiety) and the 'depside' bond (galloyl ester of gallic acid) in substrates such as tannic acids, methyl gallate and *m*-digallic acid in aqueous media and synthesize gallic acid esters with a variety of alcohols and diols in organic media [2,3].

Tannases have significant synthetic potential, as illustrated by a number of reported examples. Toth and Hensler disclosed the synthesis of gallic acid esters in the presence of tannase solved in buffer [4]. Gaathon et al. entrapped tannase in reverse micelles for transesterification of tannic with propanol to yield propyl gallate [5]. Weetal had explored to immobilize tannase on a porous silica carrier to synthesize ester in hexane containing 1-propanol and found that gallic acid yielded higher levels of propyl gallate than that in the pure alcohol system [6]. Sharma and Gupta immobilized tannase on celite-545 for transesterification of tannic acid in 1-propanol [7]. Although these synthetic studies have been performed in organic medium, very little is known about the effect of organic solvents on tannase.

In this work, *Aspergillus niger* tannase microencapsulated within chitosan–alginate complex coacervate membrane was employed to investigate the synthesis of gallic acid esters in organic solvents.

2. Materials and methods

2.1. Materials

Tannin acyl hydrolase (EC 3.1.1.20) from *Aspergillus niger* (China General Microbiological Culture Collection Center, No. 3.315) was purified by $(NH_4)_2SO_4$ fractional precipitation, DEAE-cellulose ion-exchange and finally

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freeze-dried [8]. All the chemicals were with analytical grade.

2.2. Immobilization method

A 15 mg/mL chitosan solution containing 20 mg/mL $CaCl_2$ and 2 mg/mL tannase was extruded dropwise via a syringe into a stirred 14 mg/mL alginate solution. After a reaction period of up to 20 min, the beads were collected and washed three times with distilled water, then hardened for 30 min in 20 mg/mL $CaCl_2$.

2.3. Enzymatic reactions

All reactions were carried out at 40 °C in 10 mL glass flasks shaken at 200 rpm. All solvents had been dried over molecular sieves for 72 h.

2.3.1. Synthesis of propyl gallate in water–hexane mixtures

The substrate, 1.0 mL of 0.1 mol dm^{-3} gallic acid dissolved in 1-propanol, was added into the reaction mixture, which was composed of 2 mL of 0.05 mol dm^{-3} acetic buffer (pH 5.5) and hexane. The reactions were started by adding 1 g of microencapsulated tannase to the reaction mixture.

2.3.2. Synthesis of propyl gallate in various organic solvents

The reactions were carried out by adding one gram of microencapsulated tannase to the reaction mixture, which consisted of 2 mL organic solvent and 1.0 mL of 0.1 mol dm^{-3} gallic acid dissolved in 1-propanol.

2.3.3. Enzyme specificity

The substrate, gallic acid, was dissolved in various alcohols at a final concentration of 0.1 mol dm^{-3}. One gram of microencapsulated tannase was added to 1.0 mL substrate and 2.0 mL benzene.

2.4. Analytical procedures

HPLC analysis [9] was performed on a LiChrospherC18 column (LiChroCART 125 × 4, Merck KGaA, 64271 Darmstadt, Germany). Sample (200 μL) removed from the systems was vacuum evaporated, diluted with 0.15 mmol dm^{-3} ethyl *p*-hydroxybenzoate in methanol and injected with a volume of 20 μL. The solvent system/mobile phase comprised of methanol:water in the ratio of 55:45 adjusted to pH 3 with phosphoric acid at the flow rate of 1 mL min^{-1} for 8 min. The ethyl *p*-hydroxybenzoate was as the internal standard substance. The absorbance analysis was carried out at 275 nm. The reaction product was calculated from a calibration curve plotted ($Y = 0.03144X - 0.00194$, $R = 0.9999$) as propyl gallate concentration (X, μg/mL) versus the ratio (Y) between propyl gallate peak area and internal standard substance peak area. As for synthesis of gallic acid esters, the values were determined by the disappearance of gallic acid.

Qualitative analysis was performed by thin layer chromatograph. The coating was silica gel-G plant. The solvent system was chloroform: methanol in the ratio of 8:2 with two drops of glacial acetic acid added per 100 mL of solvent. Visualization was by iodine vapor.

All the assays were done in duplicate and significantly different from tests at $P < 0.05$.

3. Results and discussion

3.1. Time-course profiles of propyl gallate synthesis in hexane

The time course of the synthesis of propyl gallate in hexane for free and microencapsulated tannase is shown as an equilibrium-controlled reaction (Fig. 1). The product synthesis both rose quickly in the first hours of incubation, and the reaction equilibrium was reached more quickly for free enzyme (4 h) than for immobilized enzyme (8 h). However, the synthetic yield with microencapsulated tannase was 19.2% higher than that with free enzyme. Many researchers had also reported that the enzyme activity was increased in organic solvent after immobilization [10–12]. Tannase microencapsulated within chitosan–alginate complex coacervate membrane was completely dissolved in the aqueous solution of vesicles. This method could not only enhance mass transfer rates of substrates and products, but also prevent direct enzyme contact with the solvent, therefore avoiding solvent toxicity. The low yield with free enzyme may be the result of inactivation by contact with the phase interface in biphasic media. However, when the water content was reduced to 1% at which Shweta et al. gained the optimum yield [7],

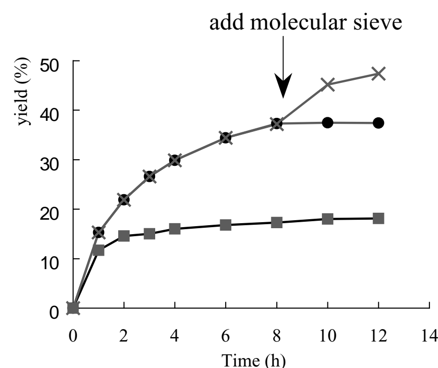

Fig. 1. Time course of propyl gallate synthesis for free (■) and microencapsulated (●) tannase and effect of water depressor (×) on tannase reaction. All reactions were performed in 1.0 mL of 0.1 mol dm^{-3} gallic acid dissolved in 1-propanol, 2 mL hexane at 40 °C, 200 rpm. The reactions were started by adding 1 g of microencapsulated tannase or the equivalent of 0.5 mg of free enzyme and 0.8 mL of acetic buffer (0.05 mol dm^{-3}, pH 5.5) to the reaction mixture. After 8 h of incubation with microencapsulated tannase, add 0.3 g molecular sieves per milliliter reaction media.

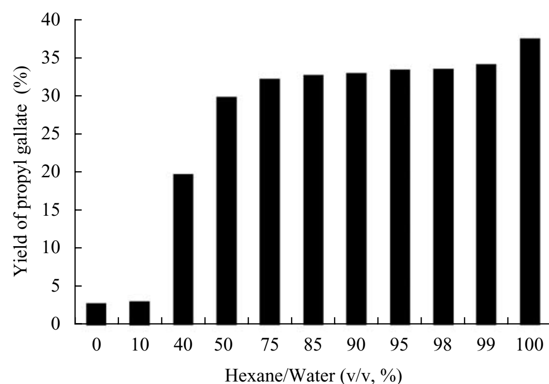

Fig. 2. Effect of water–hexane phase ratio on propyl gallate synthesis. Reaction conditions: 1.0 mL of 0.1 mol dm^{-3} gallic acid dissolved in 1-propanol, 2 mL of 0.05 mol dm^{-3} acetic buffer and hexane, 1 g microencapsulated tannase, pH 5.5, 40 °C, incubated for 12 h at 200 rpm.

the product yield was undetectable by thin layer chromatograph. It is possible to improve the activity of free enzyme by many means, such as enzyme lyophilization in the present of non-buffer salts [13], formation of enzyme–surfactant complexes [14] and control of water activity.

3.2. *Effect of hexane–water phase ratio and water depressor on propyl gallate synthesis*

In order to examine the effect of organic solvent on the yield of propyl gallate, the dependence of synthetic yield on the hexane content was investigated. Hexane (log *P*: 3.4) is commonly used in solvent systems, in which the alcohol and acid are dissolved. The yield of propyl gallate was determined at a fixed reaction time (12 h) according to the equilibrium-controlled reaction. The experimental result (Fig. 2) demonstrated that without the organic solvent, nearly no synthesis reaction occurred. However, the yield increased with raise of hexane content, especially above 75% of hexane, and reached the highest conversion of 37.3% without additional water, since organic solvent can reduce the ratio of hydrolytic reactions by lowering the water activity of the reaction media or changes the chemical equilibrium constants [15].

The level of water in the reaction system has a major effect on the equilibrium shift and the enzymatic activity. For reducing the water activity, it was carried out in this research by adding molecular sieve or anhydrous salts to the reaction system, the mechanism was the same as using glycerol, sucrose, sorbitol and NaCl to lower a_w to enhance the synthesis of the cephalosporin, cephalexin [17], which was very different from other method such as using salt hydrates to donate water to an anhydrous organic solvent [16]. The molecular sieves as water depressor could reduce the water content from reaction process or vesicle. The experiment showed that the equilibrium shifted towards esterification and the yield was improved by 10% (Fig. 1). When solid anhydrous salt was added directly to the reaction system, the anhydrous salt absorbed water from the system and converts to a high hydration state. As anticipated, the yield of propyl gallate was increased from 37.3 to 45.5% (add $MgSO_4$) and up to 49.4% (add Na_2SO_4) with the vesicles swelled slightly. While sodium carbonate, sodium acetate, and calcium oxide were added into the reaction system, the vesicles were distorted and swelled seriously, which was undesirable for industry application. The reason for swelling was that the junctions of the outer calcium alginate layer formed by an interchain chelation of calcium ions between homopolymeric blocks of L-guluronic acid residues of alginate were loosened when the binding sites of calcium ions were substituted at the present of larger amount of sodium ions or magnesium ions.

3.3. *Effect of organic solvents on propyl gallate synthesis*

Reactions in various organic solvent systems are represented in Table 1. In the case of water-miscible solvents with log *P*: below 1.0, such as *N*,*N*-dimethylformamide, acetone and ethyl acetate, very low conversions were observed for microencapsulated tannase. The data also showed that the yield was low (1.7%) only in the present of substrates, propanol and gallic acid. The low synthesis may be the result of the loss of enzyme activity caused by the removal of the essential hydration water from the enzyme by polar organic solvents, known as water stripping [18,19]. An additional possibility for synthesis decrease in polar organic solvents may be the solvent effects on substrate binding [20]. The more strongly the solvent molecules are associated with the substrate, the less favorable binding to the enzyme active site will be.

The data show the high yields obtained were 44.3 and 35.7% in benzene (log *P*: 2.0) and hexane (log *P*: 3.4), respectively, and Weetal also observed higher levels of propyl gallate synthesized in hexane than in the pure alcohol system [3], which is a refection of the enzyme stability. Water-immiscible organic solvent with high log *P* will be

Table 1
Effect of organic solvents on propyl gallate yield and log *P* values

Solvent	log *P*	Yield %
Dimethylsulfoxide	−1.3	−10.7
N,*N*-dimethylformamide	−1.0	0.3
Acetone	−0.23	0.6
1-Propanol	0.28	1.7
Ethyl acetate	0.68	2.1
Pyridine	0.71	6.0
Benzene	2.0	44.3
Hexane	3.4	35.7
Petroleumether	3.8	21.1
Octane	4.5	11.7
Dodecane	6.6	8.9

Reaction conditions: 1.0 mL of 0.1 mol dm^{-3} gallic acid dissolved in 1-propanol, 2 mL organic solvent, 1 g microencapsulated tannase, pH 5.5, 40 °C, incubated for 12 h at 200 rpm.

Table 2
Effect of alcohols on synthesis of gallic acid esters

Alkyl alcohol	C no.	log *P*	Yield %
Methanol	1	−0.76	0.85
Ethanol	2	−0.24	2.21
1-Propanol	3	0.28	44.3
Tertiary butanol	4	0.8	0.98
1-Butanol	4	0.8	37.5
1-Pentanol	5	1.31	33.8
1-Hexanol	6	1.8	1.03
1-Octanol	8	2.9	0.69
Dodecanol	12	5.0	0.65

Reaction conditions: 1.0 mL of 0.1 mol dm^{-3} gallic acid dissolved in alkyl alcohol, 2 mL benzene, 1 g microencapsulated tannase, pH 5.5, 40 °C, incubated for 12 h at 200 rpm.

less harmful to biocatalysts in organic solvent systems [21]. The relatively low product synthesis observed in octane (log *P*: 4.5) and dodecane (log *P*: 8.9) may be the result of quite low solubility of product in high hydrophobic solvents, which increase end-product inhibition in the aqueous solution of vesicles.

The experimental results demonstrated that the reaction medium exerts a strong influence on enzymatic catalysis through different mechanisms, such as the solubility of substrates and products, substrate binding and enzyme stability.

3.4. Synthesis of gallic acid esters with a variety of alcohols in organic media

The yields are shown along with the hydrophobicity of the alcohols and the optimum synthesis was observed with alcohols ranging from C_3 to C_5 with the intermediate log *P* between 0.28 and 1.31 (Table 2). The maximum yield was 44.3% of propyl gallate following 37.5% of 1-butyl gallate and 33.8% of 1-pentyl gallate. While the synthesis activity with tertiary butanol was quite low due to steric hindrance, probably its structural property is not fit for the active site of the enzyme. The low conversion with more polar alcohols such as methanol and ethanol is due to its capacity of removing water from the protein hydration shell, leading to enzyme inactivation. When increasing hydrophobicity of the reaction media, the yields decreased dramatically to 1.03% with 1-hexanol and much lower with 1-octanol and dodecanol, and almost undetected with hexadecanol and octadecanol in the solid form at reaction temperature 40 °C. Because hydrophobic alcohols with long chain ($C = 8$) could not solvate gallic acid efficiently and too bigger to establish favorable interactions with the active site of enzyme.

4. Conclusion

The combined use of organic solvent system and enzyme immobilization not only provides the advantages currently referred for free enzyme and nonconventional media [1], but also prevents enzyme from contacting directly with the solvent, therefore avoiding solvent toxicity and enhancing mass transfer rates of substrates and products. In this work, tannase from *Aspergillus niger* was microencapsulated within chitosan–alginate complex coacervate membrane and showed higher synthetic activity than that of free enzyme in organic solvent. The water content of the system can significantly affect the equilibrium shift. The synthesis of propyl gallate could be greatly improved with increasing the hexane content in water–hexane media. Adding molecular sieve or solid anhydrous salts in the reaction mixture could also improve the product yield by controlling the water activity. When the synthetic reactions were carried out in various organic solvent systems (log *P*: −1.3 to 6.6), organic solvents with the intermediate level of solvent hydrophobicity were better for synthesis of propyl gallate, probably due to the direct effect on enzyme stability, solubility of hydrophobic substrates or product and alteration of hydrolytic equilibrium [22,23]. Enzyme specificity for the alcohol portion (C_1–C_{18}) of the ester showed that the most suitable substrate was 1-propanol. The results on gallic acid esterification may reflect not only intrinsic enzyme selectivity but also the relative solubility of gallic acid and the effect on the enzyme activity due to alcohol polarity. This work allowed a better understanding of the effects of organic solvents on enzymatic synthesis of gallic acid esters and therefore it could help to define the ways to improve the performance of the synthetic reaction by immobilized tannase.

Acknowledgements

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