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ScienceDirect
Journal of Molecular Catalysis B: Enzymatic 44 (2007) 60–65
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Enzymatic galloylation of catechins in room temperature ionic liquids

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Received 9 March 2006; received in revised form 30 August 2006; accepted 11 September 2006
Available online 5 October 2006

Abstract

Galloylation (esterification with gallic acid) of catechins was achieved using a tannase from *Aspergillus niger* in room temperature ionic liquids. Immobilization of the tannase on Eupergit C substantially increased the esterification activity. Six out of seven tested ionic liquids proved adequate media for the esterification of (−)-epicatechin, with the highest yield (3.5%) in 1-butyl-3-methylimidazolium 2-(2-methoxyethoxy)-ethylsulfate. The reaction is equilibrium-controlled. Synthesis of esters was favoured with increasing concentrations of gallic acid (6.0% yield, 2 M gallic acid) and decreasing water content. However, water concentrations lower than 20% (v/v) resulted in a decrease of conversion due to inactivation of the tannase. Significant differences in the reaction yields were observed for the galloylation of epicatechin (5.4%), epigallocatechin (3.1%) and catechin (1.3%), but not for the individual (−)- and (+)-enantiomers. Tannase showed a broad specificity for the alcohol moiety and an absolute specificity for the acid portion of the ester.

Keywords: Galloylation; Catechins; Tannase; Enzymatic synthesis

1. Introduction

Tannase (tannin acylhydrolase, EC 3.1.1.20) is an esterase catalysing the hydrolysis of the ester bond of gallic acid and an alcohol moiety in substrates such as tannic acids, methyl gallate, epicatechin gallate (ECG) or epigallocatechin gallate (EGCG). In addition, it also has a so-called depsidase activity and hydrolyzes the depside bond in *m*-digallic acids [1–3].

In conventional aqueous systems, hydrolysis is thermodynamically favoured. Yet, in systems with low water activity, condensation reactions to produce an ester can be realized. Tannase has already been shown to have a considerable synthetic potential. Several reports have described the use of tannases for the synthesis of esters from gallic acid and a variety of alcohols and diols. Toth and Hensler reported the synthesis of the methyl and ethyl esters but not the phenyl ester of gallic acid in the presence of tannase dissolved in buffer [4]. Gaathon et al. reported the trans-esterification of tannic acid with propanol to yield propyl gallate by tannase entrapped in reverse micelles [5]. By immobilizing tannase on a porous silica carrier, Weetal obtained higher synthetic yields for gallic acid ester compared to the soluble enzyme [2]. Alcohols from C1 to C12 as well as several diols were found to produce gallate esters, showing a broad specificity of the tannase for the alcohol used. In contrast, absolute specificity for the acid moiety was reported. Furthermore, yields in propyl gallate were higher in a hexane propanol mixture than in the pure alcohol system [2]. Sharma and Gupta immobilized tannase on celite-545 for the synthesis of propyl gallate by trans-esterification of tannic acid [6]. Yu et al. investigated the effect of various organic solvents with microencapsulated tannase and determined the effect of the water content on the equilibrium shift [7]. Finally a mycelium-bound tannase was also employed for synthesis of propyl gallate [8]. Common to all of these synthetic studies is the use of alcohols as substrate and as solvent to solubilize gallic acid.

Recently, room temperature ionic liquids (RTIL) have emerged as alternative reaction media for biotransformations [9–11]. RTIL are salts composed of organic cations and mostly inorganic anions that do not crystallize at room temperature. They lack vapour pressure, are thermo-stable and their properties like polarity, hydrophobicity and solvent miscibility are widely tunable. Ionic liquids are of special interest for biocatalytic reactions, because unlike organic solvents of comparable polarity, they often do not inactivate enzymes, which simplifies reactions especially those involving polar substrates such as sugars [10]. Furthermore, biocatalytic reactions performed in ionic

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doi:10.1016/j.molcatb.2006.09.003

liquids have shown higher selectivity, faster rates and increased enzyme thermal stability [12–15].

Aim of this work was to carry out the galloylation of catechins and thereby increase their antioxidant potential and biological activity [16]. A major challenge realizing this novel reaction was the solubilization of the two polar substrates. The use of highly polar organic media (e.g. acetone and ethyl acetate) had already been shown to lead to inactivation of tannase. The loss of enzyme activity had been assigned to a removal of essential hydration water from the enzyme [7]. Therefore room temperature ionic liquids were employed in this work to perform the desired reaction.

2. Materials and methods

2.1. Enzymes and chemicals

Tannin acylhydrolase (EC 3.1.1.20) from *Aspergillus niger* was generously provided by Kikkoman Cooperation (Chiba, Japan).

Ionic liquids, 1-butyl-3-methyl imidazolium tetrafluoroborate [BMIM] [BF_4], 1-butyl-4-methylpyridinium tetrafluoroborate [BMPY] [BF_4], 1-butyl-3-methyl imidazolium 2-(2-methoxyethoxy)ethyl sulfate [BMIM] [$MEESO_4$], 1,3-dimethylimidazolium methylsulfate [DMIM] [MSO_4], 1-ethyl-3-methyl imidazolium tetrafluoroborate [EMIM] [BF_4], 1-butyl-3-methyl imidazolium octyl sulfate [BMIM] [OSO_4] and 1-methyl-3-octylimidazolium chloride [MOIM] [Cl] were purchased from Merck, Darmstadt, Germany. (+)-Catechin (+C), (−)-epicatechin (−EC) and (−)-epigallocatechin gallate (−EGCG) were purchased from Extrasynthese, Lyon, France. (−)-Catechin (−C), (+)-epicatechin (+EC), (−)-epigallocatechin (−EGC) and (−)-epicatechin gallate (−ECG) were from Sigma–Aldrich, Munich, Germany. All these molecules were purchased at the highest purity available.

2.2. Immobilization method

Immobilized tannase was prepared by coupling tannase oxidized with periodate to diamine-modified Eupergit C following the procedure reported by Nicolas et al. [17].

2.2.1. Periodate oxidation

Sodium periodate (0.5 ml; 0.1 M in 0.1 M sodium acetate buffer, pH 5.5) was added to a solution of Kikkoman 50000 tannase (4.5 ml; 22.22 g/l in 0.1 M sodium acetate buffer, pH 5.5). The oxidation was allowed to proceed at 0 °C for 60 min in the dark. The reaction was stopped by addition of aqueous ethylene glycol (0.5 ml; 2 M). The solution was extensively dialyzed against 0.1 M sodium phosphate buffer, pH 6.0, at 4 °C.

2.2.2. Preparation of diamine-modified Eupergit C

Eupergit C (1 g, dry) was suspended in adipic dihydrazide solution (20 ml; 0.2 M in 0.2 M sodium phosphate buffer, pH 8.0). After incubation at room temperature under mild agitation for 16 h, the modified carrier was extensively washed with distilled water and then with 0.1 M sodium phosphate buffer, pH 6.0.

2.2.3. Coupling of oxidized tannase to diamine-modified Eupergit C

Ethylene diamine-modified Eupergit C (3.48 g wet beads; 1 g dry beads) was suspended in oxidized tannase (5.43 ml) and incubated at room temperature under mild agitation for 16 h. The biocatalyst was washed with 1 M sodium phosphate buffer, pH 6.0, stored at 4 °C and filtered prior to use.

The filtered immobilized tannase had a water content of 60%. The specific activity of the immobilized tannase was determined as 38 U/g on a wet weight basis by monitoring the hydrolysis of methyl gallate in 0.1 M sodium acetate buffer at pH 6.0 and 30 °C with HPLC-UV at 280 nm. The specific activity of the soluble tannase (Kikkoman 50000) was determined as 6449 U/g using the same assay.

2.3. Enzymatic reactions

2.3.1. Standard incubation experiment

The enzymatic reactions were carried out by adding 150 mg of immobilized tannase (containing 90 μl of 1 M sodium phosphate buffer, pH 6.0) to the reaction mixture, which consisted of 280 μl ionic liquid and 20 μl 1 M sodium phosphate buffer (pH 6) containing 8.8 mM (−)-epicatechin and 365 mM gallic acid. Experiments were carried out in 2 ml eppendorf vials at 40 °C shaken at 200 rpm. After 5 h of incubation, 5 μl aliquots were withdrawn after centrifugation and added to 120 μl acetic acid (50%). Twenty-five microlitres of this mixture were then analysed by HPLC.

For the experiments with soluble tannase, 15 mg of enzyme powder (50 U/mg) was first suspended in 110 μl of 1 M sodium phosphate buffer (pH 6.0) and then added to the reaction mixture of 280 μl ionic liquid containing 8.8 mM (−)-epicatechin and 365 mM gallic acid.

2.3.2. Incubation experiment for NMR analysis of synthesis product

Four hundred and fifty milligrams of immobilized tannase were added to the reaction mixture, which consisted of 840 μl ionic liquid and 60 μl 1 M sodium phosphate buffer (pH 6) containing 88 mM (−)-epicatechin and 365 mM gallic acid. After 24 h of incubation at 40 °C the mixture was centrifuged at 10,000 × *g* for 5 min. The supernatant was withdrawn and 1 ml of 20% acetonitrile in water was added. Five hundred microlitres each of this solution were subjected to preparative HPLC. The combined fractions containing the synthesis product were lyophilised after acetonitrile had been evaporated. A total of 0.5 mg powder were obtained and analysed by NMR.

2.3.3. Synthesis of epicatechin gallate in different ionic liquids

Synthesis of epicatechin gallate was followed in standard experiments using seven different ionic liquids, [BMIM]

[BF_4], [BMPY] [BF_4], [BMIM] [$MEESO_4$], [DMIM] [MSO_4], [EMIM] [BF_4], [BMIM] [OSO_4] and [MOIM] [Cl].

2.3.4. Time-course profile of epicatechin gallate synthesis in [BMIM] [BF_4]

Four standard experiments were carried out and aliquots (5 μl) were withdrawn after 0, 5, 15, 30, 45, 60, 90, 150, 240, 330 and 1200 min and analysed by HPLC. At 1200 min, 100 μl of water was added to two of the experiments. 20.6 mg gallic acid monohydrate was added to the other two experiments. At 5, 15, 30, 60, 120, 240 and 500 min after addition, aliquots (5 μl) were withdrawn and analysed by HPLC.

2.3.5. Influence of tannase amount on initial reaction rate

Four incubation experiments were carried out with 150, 75, 37 and 0 mg of immobilized tannase in 280 μl ionic liquid and 20 μl 1 M sodium phosphate buffer (pH 6) containing 8.8 mM (−)-epicatechin and 365 mM gallic acid. To have the same amount of phosphate buffer in all experiments 45, 88 and 90 μl 1 M sodium phosphate buffer were added additionally to the experiments with 75, 37 and 0 mg of immobilized tannase, respectively.

2.3.6. Influence of water content

Six times 150 mg of filtered immobilized tannase was dried at room temperature. Afterwards, 15, 30, 40, 60, 80 and 150 μl of 1 M sodium phosphate buffer (pH 6.0) was added to the dried tannase. These tannase preparations were then incubated under standard conditions with (−)-epicatechin and gallic acid in [BMIM] [BF_4]. The final water contents of the mixtures were 5, 10, 12.5, 17.6, 22 and 35%.

2.3.7. Influence of substrate concentration

Standard experiments were performed with gallic acid concentrations of 0.03, 0.06, 0.13, 0.26, 0.51, 1.52 and 2.05 M. For the three experiments with the highest gallic acid concentrations (>0.5 M), the 20 μl of 1 M sodium phosphate buffer were replaced by 20 μl of saturated NaOH.

2.3.8. Enzyme specificity

For testing the specificity of tannase for the alcohol moiety, standard experiments were carried out and (−)-epicatechin was replaced by equimolar amounts of (+)-epicatechin, (+)-catechin, (−)-catechin and (−)-epigallocatechin, respectively. When testing the substrate specificity for the acid moiety, standard experiments were carried out and gallic acid was replaced by equimolar amounts of 2-hydroxybenzoic acid, 4-hydroxybenzoic acid, 2,4-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, 2,6-dihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 3,5-dihydroxybenzoic acid, 3-methoxy-4-hydroxybenzoic acid, 3,4,5-trimethoxybenzoic acid, cinnamic acid, 2-hydroxycinnamic acid, 3-hydroxycinnamic acid, 4-hydroxycinnamic acid and 3,4-dihydroxycinnamic acid, respectively. As a control, the same experiments were conducted in parallel without addition of (−)-epicatechin.

2.4. Analytical procedures

2.4.1. HPLC-analysis

HPLC-analysis of catechins and gallate esters was performed on a Agilent 1050 system equipped with a Nucleosil-5 C18 reverse-phase column (3 mm × 250 mm) and a diode array detector. The column was equilibrated with 95% water (containing 0.05% formic acid) and 5% acetonitrile. After injection, a linear gradient to a final solvent composition of 55% water and 45% acetonitrile was run within 12 min at a flow rate of 0.4 ml/min. Both catechins and esters were monitored by UV at 280 nm. Catechins and their esters were quantified using standard calibration curves. All enzymatically synthesized esters were identified by comparison of HPLC retention times and UV/vis spectra with authentic reference compounds run under identical conditions.

HPLC-analysis of the experiments with other acids than gallic acid was performed on the same system. A linear gradient starting with 95% water (containing 0.05% formic acid) and 5% acetonitrile to a final solvent composition of 75% water and 25% acetonitrile was run within 21 min at a flow rate of 0.4 ml/min.

2.4.2. Preparative HPLC

Purification of the synthesized product was achieved by preparative HPLC performed on a Agilent 1050 system equipped with a Vydac C18 reverse phase column (22 mm × 250 mm) and a diode array detector. The column was equilibrated with 80% water and 20% acetonitrile. After injection, a linear gradient to a final solvent composition of 40% water and 60% acetonitrile was run within 30 min at a flow rate of 5 ml/min. Catechins and esters were monitored by UV at 280 nm.

2.4.3. NMR analysis

^{1}H NMR (300.13 MHz) spectra were recorded on a Bruker DPX-360 apparatus, equipped with a 5 mm BBO gradient head, and using CD_3OD as solvent.

3. Results and discussion

Seven different ionic liquids based on dialkylimidazolium or pyridinium cations were tested as reaction media for the synthesis of epicatechin gallate from (−)-epicatechin and gallic acid with tannase immobilized on Eupergit C (Fig. 1). Six of the seven ionic liquids proved suitable media for the esterification reaction. The identity of the synthesized product was confirmed by HPLC-DAD and NMR spectroscopy after purification with preparative HPLC. The NMR data were in agreement with the data for an authentic (−)-ECg reference compound. The highest yield (3.5%) was obtained in [BMIM] [$MEESO_4$]. Four other ionic liquids ([BMIM] [BF_4], [DMIM] [MSO_4], [BMPY] [BF_4], [EMIM] [BF_4]) showed comparable synthetic yields ranging from 1.6 to 2.0%. For the experiment in 1-butyl-3-methyl imidazolium octyl sulfate [BMIM] [OSO_4] synthesis was significantly lower and for 1-methyl-3-octyl imidazolium chloride [MOIM] [Cl] formation of epicatechin gallate was not observed (Fig. 2). These results suggest that tannase activity in ionic liquid is anion dependent, as there was a lack of ester

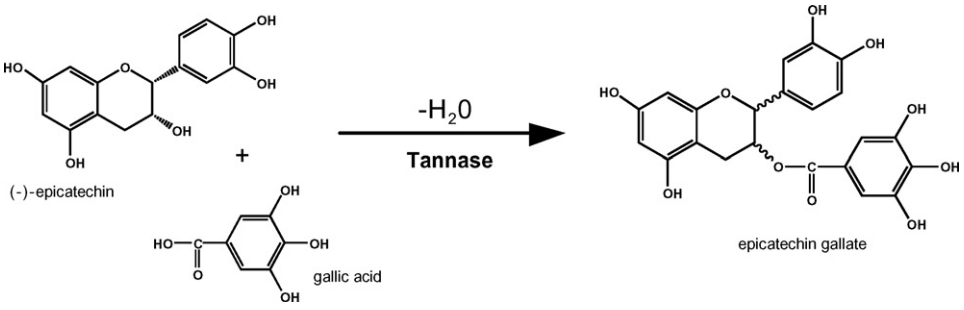

Fig. 1. Esterification of −EC with gallic acid to epicatechin gallate using tannase.

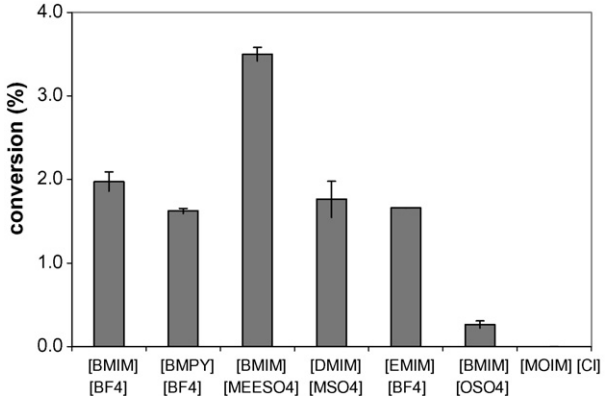

Fig. 2. Conversion of −EC to ECG by tannase immobilized on Eupergit C in different ionic liquids as determined by HPLC-UV.

synthesis in [BMIM] [OSO_4] while a significant activity was observed in [BMIM] [BF_4] and [BMIM] [$MEESO_4$] all having the same cation. Dependency of enzyme activity in ionic liquids from the anion has also been suggested by Kaar et al. [18].

Fig. 3 shows the time course of ECG synthesis in [BMIM] [BF_4] as equilibrium-controlled reaction. The maximum yield is reached after 5 h of incubation. For the experiment with soluble enzyme (total activity 96 U), a lower initial conversion rate as well as a lower final synthetic yield as compared to the immobilized enzyme preparation (total activity 5.7 U) were observed. Increased enzymatic activity in organic solvents after immobilization is a common phenomenon [19–22]. Furthermore, a higher yield for immobilized tannase compared to the free enzyme had already been reported by Yu et al. for the synthesis of propyl gallate in *n*-hexane [7].

To prove that termination of EGC synthesis was caused by reaching the thermodynamic equilibrium and not due to thermal inactivation of tannase, gallic acid and water were added separately to different experiments after 20 h of incubation. The addition of gallic acid was effective in shifting the reaction towards ester formation. The rapid rise in ECG after the addition of gallic acid demonstrates that immobilized tannase retains most of its activity even after 20 h of incubation at 40 °C in the ionic liquid/water mixture. In contrast, the addition of water led to a decrease in ECG synthesis by shifting the equilibrium towards the educts (Fig. 4).

Furthermore a direct correlation between the amount of immobilized tannase added to different incubation experiments and the initial rate of ester formation was observed (Fig. 5). In a control experiment without addition of tannase no ester synthesis was observed.

For further improvement of the esterification in [BMIM] [BF_4], the influence of water content and gallic acid concentration on the reaction yield were studied. Increasing gallic acid concentrations led to an increase in ECG formation due to a shift of the reaction equilibrium towards the products (Fig. 6). Yet, for gallic acid concentrations higher than 0.5 mol/l, the buffering capacity of the sodium phosphate buffer added to the system was exceeded. Inactivation of immobilized tannase was observed for pH values lower than 3 and thus no formation of ECG was observed. After replacement of the phosphate buffer by

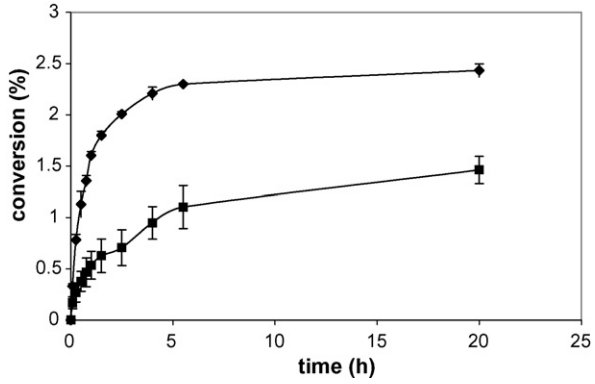

Fig. 3. Time-course profile for the synthesis of ECG in [BMIM] [BF_4] for free (■) and immobilized (♦) tannase.

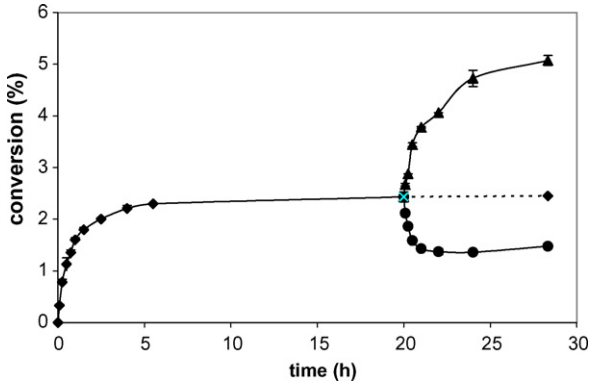

Fig. 4. Effect of the addition of gallic acid (▲) and water (●) on ECG synthesis in an incubation experiment with immobilized tannase.

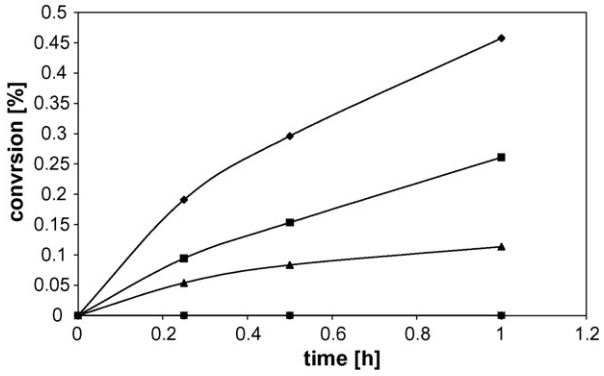

Fig. 5. Time-course profile for the synthesis of ECG in [BMIM] [BF_4] in incubation experiments with (♦) 150 mg, (■) 75 mg, (▲) 37 mg and 0 mg (●) immobilized tannase.

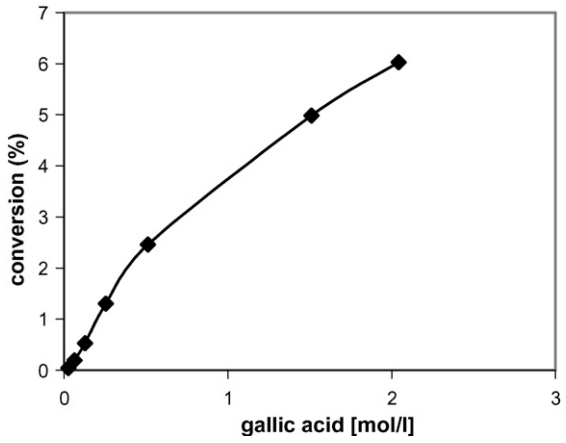

Fig. 6. Influence of the gallic acid concentration on the conversion of −EC to ECG in [BMIM] [BF_4].

the same volume of a saturated NaOH solution, a linear increase in ECG yield was demonstrated for gallic acid concentrations up to 2 mol/l.

An optimal water content of approximately 20% (v/v) was determined (Fig. 7). The decrease in yield at lower water contents seems to be the result of the loss of enzyme activity. Essential

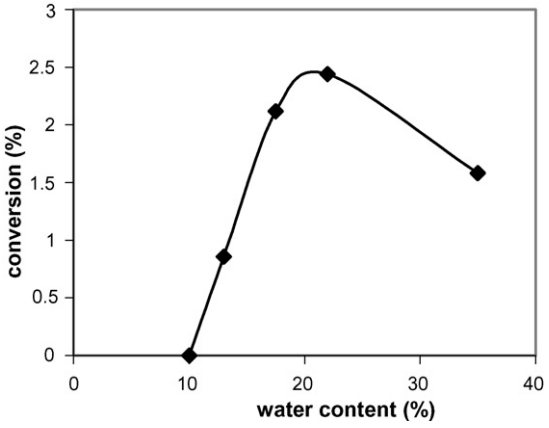

Fig. 7. Influence of the water content on the conversion of −EC to ECG in [BMIM] [BF_4].

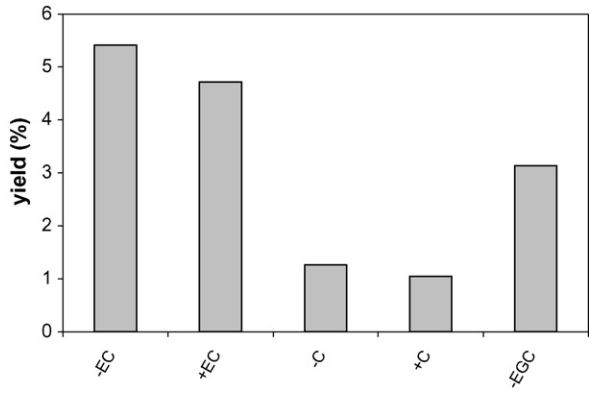

Fig. 8. Synthetic yields for the esterification of −EC, +EC, −C, +C and −EGC with gallic acid.

hydration water can be removed from the enzyme molecules by the water miscible ionic liquid, an effect known as "water stripping" [23–25]. Recently Ganske and Bornscheuer reported that Lipase B from *C. Antarctica* (CAL-B) showed no activity in the synthesis of sugar esters in pure ionic liquids [26]. Usually the activity can almost be completely regained if extra water is added to the system, but some irreversible inactivation has also been observed [27]. In our system, inactivation of the enzyme was irreversible since subsequent addition of water to the experiments with initially low water content could not reconstitute activity. Another possibility for the irreversible inactivation might be direct interactions of the ionic liquid with the enzyme [28].

Higher water concentrations than 20% (v/v) also resulted in decreased ester synthesis. The addition of water provoked an increase of water activity in the system thus shifting the thermodynamic equilibrium in favour of hydrolysis.

Substrate specificity was tested for both alcohol and acid moiety. All catechin substrates tested were converted by the tannase. Epicatechin is clearly preferred over epigallocatechin and catechin while the difference between the individual enantiomers was not significant (Fig. 8).

Beside the flavan-3-ols, also several alkyl alcohols (e.g. 1-propanol, 2-propanol, 1-butanol) were assayed under the same conditions with [BMIM] [BF_4] as solvent and were readily transformed into the corresponding gallic acid esters (data not shown).

In contrast to the alcohol, the tannase showed absolute specificity for the acid portion of the ester. By testing 14 different benzoic and cinnamic acid derivatives, no substitute for gallic acid could be found. These data are in correspondence with other reported studies. In his experiments on the ester synthesis with tannase in organic solvents, Weetal showed that tannase was specific for gallic acid [2].

4. Conclusion

The galloylation of (−)-epicatechin by tannase immobilized on Eupergit C was achieved in six different ionic liquids based on dialkylimidazolium or -pyridinium cations. Highest yields were observed for [BMIM] [$MEESO_4$].

The reaction yield was controlled by the thermodynamic equilibrium. While increasing amounts of gallic acid led to a steady increase in yield, higher water contents than 20% (v/v) resulted in lower yields by shifting the reaction towards the educts. Thus the maximum yield for ECG synthesis in [BMIM] [BF_4] was 6.0%.

A way to further increase the yield is by reducing the water activity in the system to favour ester synthesis. Yet, in case of [BMIM] [BF_4], this approach led to irreversible inactivation of enzymatic activity most likely due to stripping of essential hydration water from the enzyme.

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