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Enzymatic ester synthesis in ionic liquids

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Abstract

Six different ionic liquids based on dialkylimidazolium or quaternary ammonium cations associated with perfluorinated or bis(trifluoromethyl)sulfonyl amide anions were used as reaction media for ester synthesis catalyzed by both free *Candida antarctica* lipase B and α-chymotrypsin at 2% (v/v) water content and 50 °C. All the assayed ionic liquids proved adequate media for enzyme-catalyzed transesterification, and in the case of lipase, the synthetic activity was clearly enhanced with respect to that obtained with organic solvents of similar polarity. In general, all the ionic liquids increased the thermal stability of both the enzymes. As for example, 1-ethyl-3-methylimidazolium tetrafluoroborate enhanced 5- and 4-times the synthetic activity and half-life time, respectively, of lipase in comparison to 1-butanol.

Keywords: Ionic liquids; *Candida antarctica* lipase B; α-Chymotrypsin; Enzyme stability; Non-conventional media

1. Introduction

An active area of current research in biotechnology is biocatalysis in non-conventional media, involving all reaction systems with a reduced water content, e.g. organic solvents, supercritical fluids [1]. However, organic solvents are usually volatile liquids that evaporate into the atmosphere with detrimental effects to the environment and human health. Room temperature ionic liquids could be an alternative to organic solvents for enzyme-catalyzed synthesis in environmentally-friendly reaction media because of their negligible vapour pressure and excellent solvent properties and chemical and thermal stabilities [2,3]. Ionic liquids based on tetraalkylammonium or 1-alkyl-3-methylimidazolium cations are most interesting because it is possible to finely tune their properties by changing either the anion (e.g. BF_4^-, PF_6^-, Tf_2N^- anions) or the alkyl substituents in the cation or both as shown in Fig. 1 [4–6].

Only three of the recent papers describes the use of ionic liquids as reaction media for hydrolytic enzyme-catalyzed peptide [7] and ester synthesis [8,9], all achieving excellent results. This paper shows the transesterification activity and thermal stability of both free *Candida antarctica* lipase B and α-chymotrypsin in six different ionic liquids at low water content and 50 °C, using the synthesis of butyl butyrate and *N*-acetyl-L-tyrosine propyl ester (ATPE), respectively, as reaction models. The assayed ionic liquids were as follows: (i) 1-ethyl-3-methylimidazolium tetrafluoroborate, [emim][BF_4]; (ii) 1-ethyl-3-methylimidazolium bis[(trifluoromethyl)sulfonyl]amide or triflimide, [emim][Tf_2N]; (iii) 1-butyl-3-methylimidazolium hexafluorophosphate, [bmim][PF_6]; (iv) 1-butyl-3-methy-

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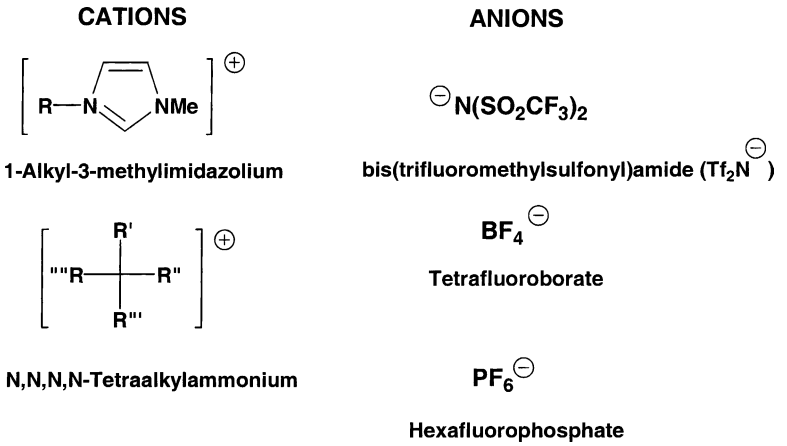

Fig. 1. Common ions involved in ionic liquids.

limidazolium tetrafluoroborate, [bmim][BF_4]; (v) 1-buty-l-3-methylimidazolium triflimide, [bmim][Tf_2N]; and (vi) methyltrioctylammonium triflimide [mtoa][Tf_2N].

2. Experimental

2.1. Materials

Soluble *Candida antarctica* lipase B (Novozym 525, EC 3.1.1.3, from Novo Nordics S.A.) was washed by ultrafiltration to eliminate all the low molecular weight additives, obtaining a *C. antarctica* lipase B solution of 5.74 mg ml^{-1}, as determined by the Lowry's method. α-Chymotrypsin (EC 3.4.21.1) type II from bovine pancreas, substrates, solvents and other chemicals were purchased from Sigma–Aldrich–Fluka Chemical Co., and were of the highest purity available.

2.2. Synthesis of ionic liquids

1-Ethyl-3-methylimidazolium tetrafluoroborate [emim][BF_4] and 1-butyl-3-methylimidazolium tetrafluoroborate, [bmim][BF_4] were prepared according to the procedure described in [10]. 1-Ethyl-3-methylimidazolium triflimide [emim][Tf_2N] and 1-butyl-3-methylimidazolium triflimide [emim][Tf_2N] were synthesized following the procedure described in [4]. 1-Butyl-3-methylimidazolium hexafluorphosphate, [bmim][PF_6] was prepared according to the procedure described in [11]. Methyl trioctylammonium triflimide, [mtoa][Tf_2N] was prepared as follows: 15 mmol of aliquat 336 (6.06 g) and 15 mmol of $LiNTf_2$ (2.31 g) were dissolved in 75 ml of acetone at room temperature. After 24 h, the reaction mixture was filtered through a plug of celite. The solvent was removed under reduced pressure and the ionic liquid dried under high vacuum (10^{-2} Torr) at 70 °C for 24 h giving 4.37 g of a clear oil (85% molar yield). ^{1}H NMR (acetone (d_6, 300 MHz, δ ppm/TMS) δ: 3.49 (m, 6H), 3.21 (s, 3H), 1.90 (brs, 6H), 1.38 (m, 36H), 0.88 (t, $J = 6.6$ Hz, 9H). ^{13}C NMR (acetone, 50 MHz, δ ppm/TMS) δ:123.51, 118.52, 92.48, 62.52, 32.59, 32.41, 26.48, 23.30, 23.23, 22.78, 14.32. Positive-ion LSIMS (*m*/*z*): 1014.8, 1042.9, 1070.8, 1098.9, 1126.9, 1154.8.

2.3. α-Chymotrypsin transesterification activity

An amount of 125 μl of 0.8 M *N*-acetyl-L-tyrosine ethyl ester (ATEE) solution in 1-propanol containing 0.4 M triethylamine, were added to screw-capped vials of 1 ml total volume. Then, 855 μl of each ionic liquid, [emim][BF_4]; [emim][Tf_2N]; [bmim][BF_4]; [bmim][PF_6]; and [mtoa][Tf_2N] were added, and the resulting solution was homogenized. The reaction was started by adding 20 μl of 1 mg ml^{-1} of α-chymotrypsin in water and run at 50 °C with shaking

for 1 h. At regular time intervals, 25 μl aliquots were extracted and dissolved in 475 ml of 50% (v/v) acetic acid to stop the reaction by vigorous shaking for 5 min. Substrate and product concentrations were determined by HPLC using a LiChrospher RP-18 column (15 cm length and 3.9 mm i.d., 5 μm particle size and 10 nm pore size). Samples were eluted using a linear gradient (phase A: 10% (v/v) acetonitrile and 1% (v/v) acetic acid; phase B: 50% (v/v) acetonitrile and 1% (v/v) acetic acid) at 1 ml min^{-1} flow rate. Elution profiles were monitored at 276 nm. One unit of synthetic activity was defined as the amount of enzyme that produces 1 μmol of *N*-acetyl-L-tyrosine propyl ester per minute. All experiments were carried out in duplicate.

2.4. Lipase transesterification activity

To screw-capped vials of 1 ml total capacity, 300 μl of each ionic liquid ([emim][BF_4]; [emim][Tf_2N]; [bmim][PF_6]; and [bmim][Tf_2N]), or hexane, or 1-butanol, 30 μl (236 μmol) of vinyl butyrate and 110 μl (1.21 mmol) of 1-butanol were added. The reaction was started by adding 10 μl of 2.86 mg ml^{-1} *C. antarctica* lipase B in water and run at 50 °C in oil bath with shaking for 1 h. At regular time intervals, 20 μl aliquots were taken and suspended in 1 ml of hexane. The biphasic mixture was strongly shaken for 3 min to extract all substrates and product into the hexane phase. For hexane and 1-butanol reaction media, aliquots were dissolved in acetone:HCl (99:1 (v/v)) to stop the reaction. Then, 400 μl of the hexane or acetone extracts were added to 600 μl of 10 mM propyl acetate (internal standard) solution in hexane, and 1 μl of the resultant solution was analyzed by GC. Analysis were performed with a Shimadzu GC-17A instrument equipped with a FID detector and a Nukol™ column (15 m × 0.53 mm, Supelco), using the following conditions: carrier gas (nitrogen) at 8 kPa (20 ml mm^{-1} total flow); temperature program: 45 °C, 4 min, 8 °C min^{-1}, 133 °C; split ratio: 5:1; detector, 220 °C. Retention time of peaks were as follows: propyl acetate, 3.2 min; vinyl butyrate, 4.3 min; 1-butanol, 6.6 min; butyl butyrate, 7.7 min; and butyric acid, 13.5 min. One unit of synthetic activity was defined as the amount of enzyme that produces 1 μmol of butyl butyrate per mm. All experiments were carried out in duplicate.

2.5. Enzyme stability

Into different screw-capped vials, 400 μl of solvent (hexane, 1-propanol, 1-butanol) or ionic liquids ([emim][BF_4]; [emim][Tf_2N]; [bmim][PF_6]; [bmim][Tf_2N]; [bmim][BF_4]; [bmimj[PF_6]; or [mtoa][Tf_2N],) and 10 μl of enzyme solution (2.86 mg ml^{-1} *C. antarctica* lipase B or 1 mg ml^{-1} chymotrypsin) in water, were added, and the mixtures incubated at 50 °C. Then, at selected incubation time, 90 μl of substrate solution (1.68 M vinyl butyrate in 1-butanol for lipase, and 0.8 M ATEE in 1-propanol with 0.4 M triethylamine for chymotrypsin, respectively) were added to each vial, and the reaction was followed as described above for each enzyme. All experiments were carried out in duplicate.

3. Results and discussion

Fig. 2 shows the synthetic activity exhibited by both lipase and α-chymotrypsin in the different assayed ionic liquids. Enzyme reactions in organic solvent systems were also carried out to compare the efficiency of ionic liquids for use as reaction media. In all the cases, ionic liquids proved suitable media for both the enzyme-catalyzed transesterification, except the [emim][BF_4] medium for α-chymotrypsin. For lipase-catalyzed butyl butyrate synthesis (Fig. 2A), in all the cases, the initial synthetic rate was higher than that in both assayed organic media (1-butanol and hexane), the best results being obtained with [emim][BF_4]. Additionally, the use of a low water content meant that the selectivity parameter was almost 100% for all the ionic liquids, being slightly higher in ionic liquids than that in organic media. However, for α-chymotrypsin-catalyzed ATPE synthesis (Fig. 2B), the enzyme activity in 1-propanol was higher than that in ionic liquids, the selectivity parameter being near to 85% in all the cases. Furthermore, as can be seen in Fig. 2, the increase in both the chain length of the alkyl substituents of cation and the anion size was involved in a decrease in both lipase and α-chymotrypsin synthetic activities.

Ionic liquids have been described as solvents with a polarity comparable to that of the lower alcohols (e.g. methanol, ethanol, 1-butanol, etc.) [4,5,12,13]. Thus, Carmichael and Seddon [12] reported a correlation

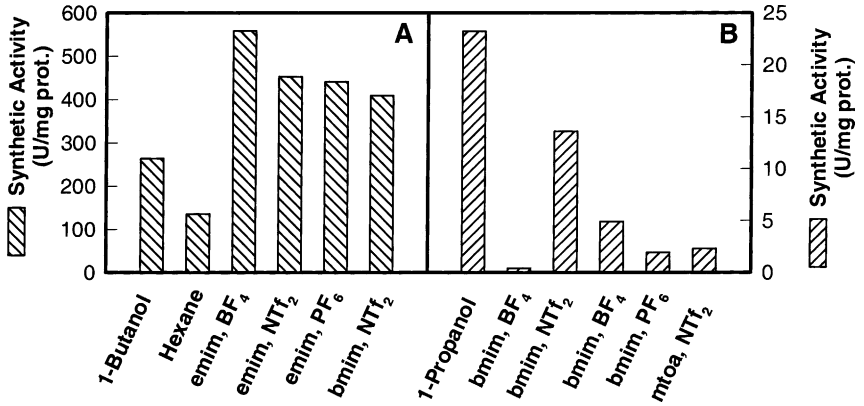

Fig. 2. Synthetic activity of both *C. antarctica* lipase B to produce butyl butyrate (A) and α-chymotrypsin to produce *N*-acetyl-L-tyrosine propyl ester (B) in different organic solvents and ionic liquids at 2% (v/v) water content and 50 °C.

between the decrease in both the chain length of the alkyl substituents on the imidazolium ring of cation and the anion size with the increase in polarity. Being this in mind, the increase in polarity of the ionic liquids could be involved in the increase in both enzymes-catalyzed transesterification.

To ascertain the influence of these new reaction media on the enzyme, the stability of both lipase and α-chymotrypsin was analyzed by incubating the enzymes in the assayed ionic liquids and organic solvents, respectively, at 50 °C and 2% (v/v) water content. In all the cases, the kinetics of enzyme deactivation followed a one-step first-order deactivation function, from which the half-life time ($t_{1/2}$) of both the enzymes are depicted in Fig. 3 can be determined. As can be seen from Fig. 3A, the half-life time of lipase decreased slightly with a decrease in polarity, the best results again being obtained for the most polar ionic liquid ([emim][BF_4]). The behaviour of α-chymotrypsin was clearly different Fig. 3B), because the decrease in polarity of the ionic liquids produced an increase in its half-life time. These results are in accordance with the previous works [14,15], where α-chymotrypsin was very sensitive to deactivation by the increase in polarity of organic solvents at low water content. Thus, the half-life time of α-chymotrypsin in the ionic liquid of lowest polarity ([mtoa][NTf_2] was 13.5-times higher than that in 1-propanol.

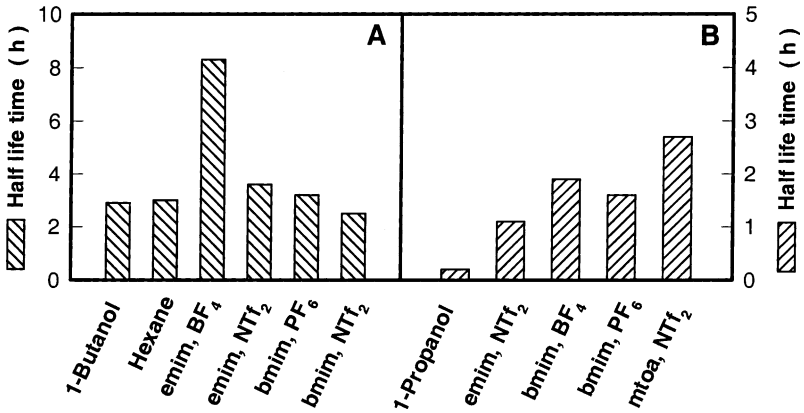

Fig. 3. Half-life times of both *C. antarctica* lipase B (A) and α-chymotrypsin (B) in different organic solvents and ionic liquids at 2% (v/v) water content and 50 °C.

In conclusion, ionic liquids are shown to be excellent media for both chymotrypsin and lipase-catalyzed transesterification reactions, compared with common organic media. Additionally, ionic liquids are good stabilizing agents, which prevent thermal deactivation of the enzyme. The inclusion of free enzyme in the ionic liquid matrix may provide the protein with a conformation more suited to synthetic activity. Ionic liquids appear to be a clean alternative to organic solvents for biocatalysis in non-conventional media.

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