Preparation of CLA ascorbyl ester with improved volumetric productivity by an ionic liquid-based reaction system

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A new approach to the enzymatic production of conjugated linoleic acid (CLA) ascorbyl ester with a remarkably high volumetric productivity (120–200 g L^{-1}) has been developed, in which strong solvation by *t*OMA·TFA (methyltrioctylammonium trifluoroacetate) enables a high concentration of ascorbic acid to be applied, and in which *t*-butanol enhances conversion by changing the equilibrium constant of the activity coefficients. This work has experimentally demonstrated the practicability of achieving efficient reactions of polar compounds at high concentrations in ionic liquids without significant loss of enzyme activity.

Introduction

Ionic liquids (ILs) have been introduced as solvents into synthetic chemistry to meet the increasing demand for clean technologies in industrial processes.[1] Thanks to the possibility of tuning their properties by modifying the anions or the cations that comprise these liquid salts, ionic liquids are considered as the ideal tailorable solvents.[2] Biocatalysis in ILs represents an exciting area that couples environmentally benign biocatalytic approaches with these new green solvent.[3] It is known that many enzymatic processes operate under conditions where the position of chemical equilibrium may control the final yield achieved; therefore, to select or adjust the property of solvents in which the enzyme operates is a crucial factor in enhancing the conversion and product selectivity.[4] Ionic liquids compensate for the lack of conventional solvents that are capable of dissolving a wide range of compounds, and in addition offer the advantage of being able to be adjusted to a particular application.[3,5] However, successfully utilising the advantages of ILs depends primarily on formulating a correct description of the multiple interactions between substrates, products, ILs and enzymes, as well as the subsequent strategy. Recently, we demonstrated an approach based on COSMO-RS (conductor-like screening model for real solvents) that could be employed in the fast screening of ILs with regard to their ability to solubilise compounds with multiple hydroxyl groups.[6] Ascorbic acid (Vitamin C) falls into this category (Scheme 1) and therefore could benefit from this approach for establishing an efficient enzymatic esterification system.

Since the fatty acid esters of ascorbic acid find attractive applications in cosmetics, pharmaceuticals and food ingredients, a lot of effort has been made in the enzymatic preparation of these lipophilic derivatives in order to establish a protocol with high volumetric productivity.[7] However, the advances are limited by the low solubility of ascorbic acid in conventional solvents. Park *et al.*[8] reported the first successful attempt at using ILs to host lipase-catalyzed esterification of ascorbic acid at relatively high concentrations of ascorbic acid. In this paper, we report our progress in setting up a highly productive reaction protocol for the enzymatic preparation of fatty acid ascorbyl esters, using conjugated linoleic acid (CLA) (which has multiple physiological activities and antioxidation potential) as a model fatty acid[9] (Scheme 1). This work attempts to verify the practicability of the following technology evolution: COSMO-RS *a priori* screening of commercially available ionic liquids, experimental validation of screened systems, and protocol optimization based on a correct understanding of multiple solvation interaction delineation by COSMO-RS.

Results

Solvent dependency of reaction behaviours

As recently demonstrated, the solubility of the analogues with multiple hydroxyl groups as H-bonding donors in ILs is largely determined by the property of anions of ILs, and depending on the basicity of the anions of ILs can be categorized into 3 groups.[6] Based on these results, it would seem not to be too difficult to identify commercially available ILs that can dissolve ascorbic acid at high concentrations; however, most of these IL candidates frustrate the activity of enzymes due to the strong coordinating capacity of the anions.[8] Those ILs in which enzymes *can* work well are incapable of dissolving ascorbic acid at a high concentration. Therefore, the judicious selection of ILs is imperative, and the COSMO-RS predictions[5,6] and experimental results suggested that we should focus on those ILs with considerable dissolving ability but less coordinating capacity (Table 1). In our attempt to obtain the results in Table 1, we have screened over 20 ILs with different anions and cations. No detectable reaction was observed in alkylimidazolium- and alkylammonium-type ILs with the anions of 2-(2-methoxyethoxy)ethylsulfate ($MDEGSO_4^-$), *n*-octylsulfate ($OctSO_4^-$), ethylsulfate ($EtSO_4^-$), dimethylphosphate ($Me_2HPO_4^-$) and toluene-4-sulfonate (OTs^-). With paired anions of tetrafluoroborate (BF_4^-), hexafluorophosphate (PF_6^-)

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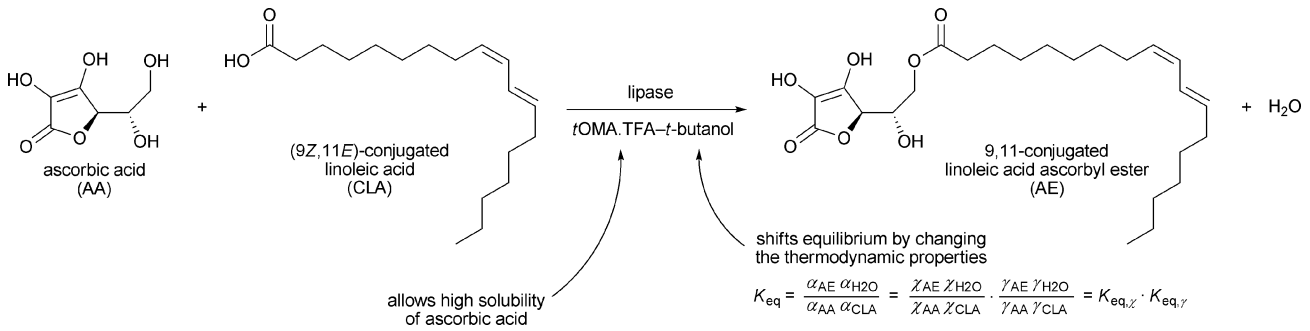

Scheme 1 Schematic representation of enzymatic synthesis of CLA ascorbyl ester (AE) from ascorbic acid (AA) and (9*Z*,11*E*)-conjugated linoleic acid (CLA) in *t*OMA·TFA–*t*-butanol and the corresponding equilibrium equation. a_i, χ_i and γ_i represent the thermodynamic activity, mole fraction and activity coefficient of component *i*, respectively. $K_{eq,\chi}$ and $K_{eq,\gamma}$ are the corresponding constants at mole fraction χ and activity coefficient γ, respectively.

and bis(trifluoromethylsulfonyl)imide (Tf_2N^-), we could achieve similar conversions to those observed by Park *et al.* when lower concentrations (around 20 mg mL^{-1}) were employed.[8] However, when higher concentrations was used, <5% conversion of ascorbic acid after 24 h was observed in BMIM·BF_4, BMIM·PF_6 and *t*OMA·Tf_2N (data not shown). It should be pointed out that in this work the concentration of ascorbic acid applied (1.22 mmol mL^{-1} IL) is significantly higher than previous reports for conventional solvents (0.1–0.3 mmol mL^{-1} solvent)[7] or the ILs examined (200 mM).[8] There has been concern that the initial reaction rate is controlled by the amount of substrates dissolved and/or dissolution rate.[8,10] The increase in conversion of ascorbic acid in going from *t*-butanol to *t*OMA·TFA shows a strong correspondance with the solubility (Table 1). The solubility of ascorbic acid in *t*OMA·TFA is around 3-fold that in *t*-butanol – coincidentally the same as the ratio of the conversions (Table 1); the conversion of ascorbic acid in BMIM·CF_3SO_3 also seems to match the solubility. Compared with the conversion in *t*-butanol (one of the best solvents for bioconversion of ascorbic acid),[7] the result of *t*OMA·TFA is pretty encouraging.

However, this method is still some way from being a high volumetric productivity protocol due to the low conversion, even though high substrate concentrations can be applied. Park *et al.* ascribed the lower conversion of ascorbic acid in their systems to the inhibiting action of the product accumulating around the lipase, an effect that can be avoided by adding hydrophobic solvents.[8] We therefore added hexane (entry 4) and toluene (entry 5); an evident enhancement of conversion was obtained (Table 1). With this observation and the COSMO-RS prediction, we anticipated that this could be also applicable for other solvents. This was verified by the result shown in entry 6, where over 70 mol% conversion of AA (86% by weight) was achieved when an equal volume of *t*-butanol was mixed with the IL. For BMIM.CF_3SO_3 the addition of *t*-butanol results in a conversion increase by a factor of 1.5. Fig. 1 depicted the time course of enzymatic esterification of ascorbic acid in *t*OMA·TFA with or without solvent addition.

Clearly, the addition of all solvents results in a marked increase of initial rate (Fig. 1). In pure *t*OMA·TFA, little reaction was detected in the first 1 h, while the presence of *t*-butanol gave 9.5% conversion. Mass transfer hindrance could be a reason for the lower initial rate in pure *t*OMA·TFA due to its high viscosity;[12] therefore this might also represent one of the leading reasons for the elevated initial rate, since the introduction of organic solvents

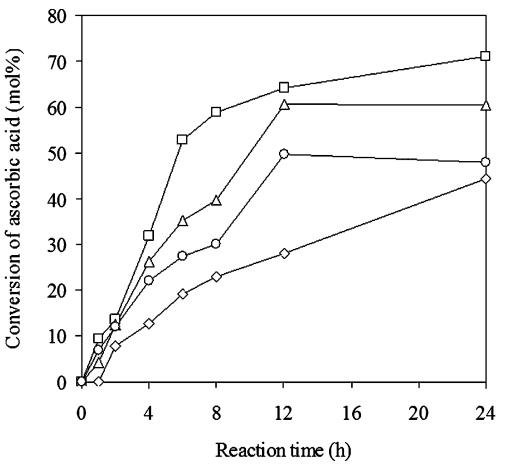

Fig. 1 Effects of solvents on enzymatic esterification of ascorbic acid in methyltris(octyl)ammonium trifluroacetate (*t*OMA·TFA). Reaction conditions: 1.22 mmol ascorbic acid (0.2148 g) and 6.1 mmol CLA (1.714 g) were dissolved in 0.5 mL IL and 0.5 mL *t*-butanol (□), heptane (△) or toluene (○), or just 1 mL IL on its own (◇). The reaction was conducted in a jacketed reactor at 70 °C and 300 rpm, in the presence of 200 mg activated molecular sieves and 200 mg of Novozym 435.

leads to a viscosity reduction of the system of the same order of magnitude as *t*-butanol (Table 1). In the first 6 h, almost all systems with organic solvents show a similar linear increase, but with different final conversions (*t*-butanol 53% and toluene only 27%). In the systems containing hexane and toluene, the stabilization in the conversion after 12 h suggests that thermodynamic equilibrium is being reached; while the reaction in the IL with *t*-butanol continues to undergo a steady increase (Fig. 1). Another test showed that prolonging the reaction time only leads to a slight increase of conversion in *t*OMA·TFA (45–50%) or its mixture with *t*-butanol (70–75%) (data not shown).

Important parameters to affect volumetric productivity

Fig. 2A reveals how ascorbic acid concentrations affect AA conversion and volumetric productivity of CLA ascorbyl ester. In terms of conversion, the optimum concentration of ascorbic acid is 65–68 g L^{-1}, and increasing the substrate loading further results in a decrease in conversion. This optimum of concentration has also been observed in a similar reaction, which is suggested to reflect the change in the lipase/substrate concentration ratio.[7e] However, the

Table 1 Comparison of enzymatic production of CLA ascorbyl ester in ionic liquids or in mixtures of ILs with conventional solvents[a]

Entry	Solvent	Predicted solubility at 25 °C (g per 100 g)[b]	Measured solubility at 60 °C (g per 100 g)[c]	Viscosity/mPa s[d]	Conversion (mol%) after 24 h	Volumetric productivity of CLA ascorbyl ester/g L^{-1}	K_{eq}
1	*t*OMA·TFA	> 15	15.44	2246.13	44.44	75.15	36.67
2	BMIM·CF_3SO_3	5.83	6.82	115.18	31.32	52.94	3.41
3	*t*-Butanol	4.99	4.63	4.31	16.25	27.49	3.56
4	*t*OMA·TFA–heptane	—	—	1.99	60.42	102.17	18.12
5	*t*OMA·TFA–toluene	—	—	1.25	48.01	81.18	21.11
6	*t*OMA·TFA–*t*-butanol	—	—	5.32	71.01	120.07	7.76
7	BMIM·CF_3SO_3–*t*-butanol	—	—	6.58	46.03	77.84	3.87

[a] The reactions and sample analysis follow the typical procedure described in the Experimental section. [b] COSMO-RS-computed solubility is a zeroth order approximation, which is valid only for small concentrations of the solute. For the solubility of AA in *t*OMA·TFA (>0.1 molar fraction) it is a poor estimation. [c] The measurement of the corresponding solubilities at 60 °C by HPLC analysis was carried out as previously, with some modifications.[6] UV detection at 254 nm instead of ELSD was employed for quantification of the concentration of ascorbic acid. [d] Viscosity values of ILs (at 20 °C) used were provided by the suppliers. The IL–solvent mixtures were treated as ideal systems in the estimation of viscosity by the Grunberg correlation.[11]

volumetric productivity of CLA ascorbyl ester steadily increases as substrate concentration increases, reaching 130 g L^{-1}. It is worth noting that the concentrations of ascorbic acid shown in Fig. 2A are based on total volume of solvent and substrates, in which the volume of CLA occupies a larger proportion due to the excess CLA applied. We therefore tested the reaction with various CLA/AA mole ratios (1 : 1 to 10 : 1) using *t*OMA·TFA–*t*-butanol (1 : 1, v/v) (Fig. 2B). As can be seen, at an optimum substrate ratio of CLA/AA 5 : 1, 74.5% conversion of AA could be achieved when the reaction proceeds for 36 h. At lower substrate ratios (CLA/AA from 1 : 1 to 5 : 1), the conversion rate of AA increases steadily with increasing CLA dosage, while a decline appears when the CLA/AA ratio is increased above 5 : 1. However, as shown in Fig. 2B, the volumetric productivity seems to be unaffected by the changing AA conversion. This result indicates that, in such concentrated reaction systems, the volumetric productivity is dependent on the total volume change rather than small changes in conversion, since concentration variation of a substrate with a larger molar volume (like CLA) would significantly change the total volume of reaction mixture, thereby influencing the volumetric productivity. It was also observed that the conversion rate *vs.* substrate ratio is time-dependent; namely, the reactions with higher CLA concentrations (CLA/AA 5 : 1 or higher) are much faster. The equilibrium can be achieved around 24 h, whereas, the equilibrium for the reaction with CLA/AA 1 : 1 was achieved after 36 h. This result reflects the substantial influence of the substrate on the property of the reaction medium in a highly concentrated system, as well as its subsequent effect on enzymatic reactions. Although higher volumetric productivity for CLA ascorbyl ester (over 200 g L^{-1}) at a lower ratio of CLA/AA (Fig. 2B) could be obtained, it needs a longer time and also results in a larger proportion of unconverted AA. Therefore, considering the overall efficiency of the protocol, using a CLA/AA ratio of 4 : 1 to 6 : 1 would be preferable, giving a volumetric productivity of 120–150 g L^{-1} after 36 h.

Effects of temperature and lipase dosage were also examined in this work, and both were found to affect the reaction rate profoundly. However, the conversion at equilibrium is not significantly altered if the reaction proceeds for the proper time. The optimum temperature and Novozym 435 concentration were found to be 70 °C and around 200 mg mL^{-1} solvent, respectively, and these values were thus employed in the these studies (Fig. 1 and 2).

Concerning the separation of product, we found that the product could be precipitated by adding water after unreacted CLA was removed by hexane extraction, as previously reported.[8] Further investigations on product recovery and IL reusability are in progress. The identification of the product in this work was conducted by a model reaction of palmitic acid with ascorbic acid, which has been well characterized previously.[7d,8] In agreement with these studies, only one product was obtained, which was identified as 6-*O*-L-ascorbyl palmitate. CLA 6-*O*-L-ascorbyl ester (only one product peak) was therefore identified by comparison of retention time with 6-*O*-L-ascorbyl palmitate in HPLC analysis.

Discussion

A solvent reaction system with high volumetric productivity relies on high solubility and good conversion, both of which depend mainly on the properties of the medium.[4] The highly

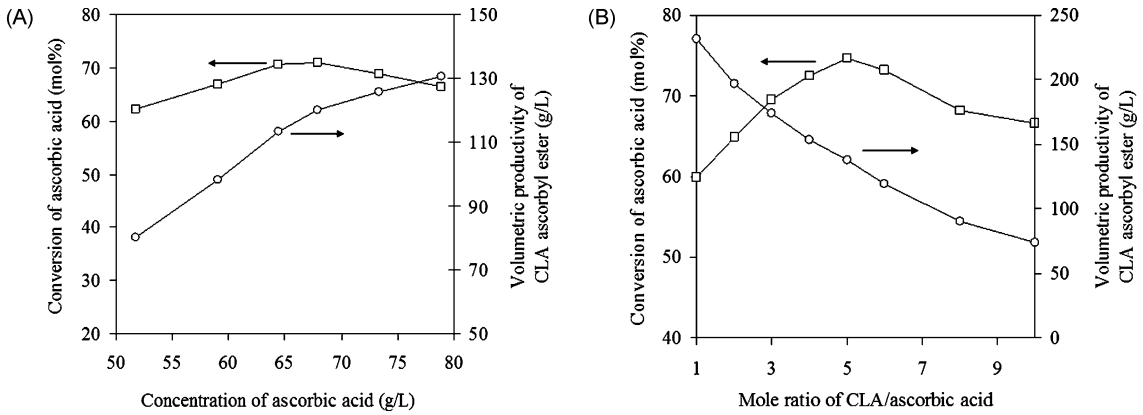

Fig. 2 Effects of substrate concentrations and substrate ratios on the conversion of ascorbic acid and volumetric productivity of CLA ascorbyl ester in *t*OMA·TFA–*t*-butanol. All reactions were conducted in a mixture of 0.5 mL *t*OMA·TFA and 0.5 mL *t*-butanol. (A) A fixed CLA/AA mole ratio (5 : 1) was applied, and the data shown acquired after 24 h. (B) The CLA/AA mole ratio was altered but the dosage of ascorbic acid was fixed at 1.22 mmol, and the data shown acquired after 36 h. Other conditions are the same as in Fig. 1.

substrate-concentrated reaction system presented in this work, with better conversion (over 70%) and remarkably high volumetric productivity (120–200 g L^{-1}), can be principally attributed to the good solubility of ascorbic acid in *t*OMA·TFA[10] (Table 1). However, high solubility doesn't naturally yield a good reaction if the property of solvent doesn't favour the formation of products (Scheme 1). Sometimes the activity coefficients of reactants or products, resulting from different interactions with the solvents, can profoundly influence the selectivity and product yield by shifting the reaction equilibrium.[5,12] We have experimentally proven that employing a lower concentration of ascorbic acid or prolonging the reaction time do not significantly increase the conversion in pure *t*OMA·TFA. The effect of fatty acid solubility and product inhibition could be also ruled out because both are experimentally observed to be soluble in *t*OMA·TFA.[8] A possible reason for the significant effects of organic solvents added to *t*OMA·TFA on the conversion of ascorbic acid is that the equilibrium is changed due to activity coefficient change of substrates and products in the resulting mixed system, which has been demonstrated in many enzymatic reactions.[4,12] Unfortunately the direct measurement of activity coefficients of solids (herein ascorbic acid) is methodologically difficult in ILs, but they can be estimated by an independent and physically well-founded model, COSMO-RS.[14] It has been known that for a specific reaction at a certain temperature the equilibrium constant of thermodynamic activity is a constant and is equal to $K_{eq,\chi}K_{eq,\gamma}$ (Scheme 1); therefore, it is possible to shift the equilibrium to esterification (increasing $K_{eq,\chi}$ value) by reducing the equilibrium constant of the activity coefficients $K_{eq,\gamma}$ (Scheme 1).[4,5] Table 1 shows COSMO-RS-computed $K_{eq,\gamma}$ values based on infinite dilution of substrates and products in individual solvent systems.[13] Interestingly, all $K_{eq,\gamma}$ values marked decreased when equal volumes of conventional solvents are added to *t*OMA·TFA; *t*-butanol gives the most significant reduction (from 36.67 to 7.76), following hexane (18.12) and toluene (21.11). Surprisingly, this trend agrees with the corresponding enhancement of the conversion of ascorbic acid (from 44.44% to 71.01, 60.42 and 48.01%, respectively). Of course, estimates of infinite dilution-based activity coefficients are not sufficient to quantitatively correlate with equilibrium shifts, but at least COSMO-RS is capable of giving a theoretically correct qualitative evaluation on how the property change of a medium affects equilibrium shifting.[5,13] This is very useful for better understanding the reaction behaviours in different media or pre-evaluating a single or mixed solvent system.

It should be realized that direct thermodynamic computation of such a concentrated system using the current models will greatly deviate from the real values, because the strongly non-ideal thermodynamic behaviour in this system is beyond the effective range of the model.[14] Despite this limitation, as mentioned earlier, computations of solubility and activity coefficients based on indefinite dilution-mode is still of great instructive value to clarify some issues that are currently impossible by other methodologies.

In actual fact, the reaction behaviour in *t*OMA·TFA and its mixtures with organic solvents at high substrate concentrations can also be explained from an understanding of the mechanism. There is no doubt that the strong coordinating capacity of trifluoroacetate and its interaction with H-bonding donors in ascorbic acid is the main reason for the high solubility of AA in *t*OMA·TFA. The question is, then, how this coordination will affect lipase activity. In the pure ionic liquid reaction system, we do not know whether the lower enzyme activity or the thermodynamically unfavourable formation of the product will result in a lower conversion of AA at equilibrium. To address these questions, we conducted several control tests. It turns out that the lipase activity is indeed influenced by the coordination interaction in *t*OMA·TFA; therefore, the introduction of some less-coordinating solvents generally leads to an increase of initial reaction rate (Fig. 1). However, it is found that the lower enzyme activity in IL is not the main reason leading to a lower final conversion of AA, because employing excessive biocatalyst, prolonging the reaction time, or the batchwise addition of enzyme (to keep it continuously available for reaction), didn't significantly improve the conversion of AA at equilibrium in the pure IL system. In fact, the influence of the added solvents on the equilibrium shifting can be understood from a thermodynamic point of view. The strong interaction between substrate (AA) and *t*OMA·TFA heavily restricts the freedom (activity) of substrate, which is essential for an effective collision leading to reaction, while the moderate property change

resulting from the introduction of organic solvents (*i.e.* becoming more hydrophobic) could counteract the above effect and make the substrate more active. On the other hand, the product (CLA ascorbyl ester) becomes less active (negative for the reverse reaction) with the introduction of organic solvents because the product is more hydrophobic compared with substrate (AA) and a stronger interaction occurs within a more hydrophobic environment. Thus both effects facilitate the shifting of the equilibrium towards the formation of CLA ascorbyl ester, corresponding to a higher conversion of AA at equilibrium (Scheme 1 and Table 1).[5,10] With these hypotheses, one can understand that the contribution of substrate concentration to the conversion rate in such concentrated system doesn't resemble a normal solvent reaction system, in which the property change of the reaction system with the addition of substrate can be neglected. Substantial addition of CLA (with a larger molecular volume) will change not only the total volume but also the property of the reaction system (Fig. 2A). Similar effects might be generated by varying the CLA/AA ratio (Fig. 2B). Therefore, without consideration of other effects, the increase of CLA dosage generally results in an increased conversion rate. However, overload of CLA will dramatically decrease the dissolution of AA in the resulting mixture, leading to a decreased reaction rate. Possibly this might be one of the reasons why an optimum appears in either substrate concentrations (Fig. 2A) or CLA/AA ratios (Fig. 2B) in terms of the conversion of AA.

Conclusions

In summary, we have demonstrated an efficient IL reaction system for the production of fatty acid ascorbyl esters with very high throughput (120–200 g L^{-1}). The strong solvation of *t*OMA·TFA allows a high concentration of ascorbic acid to be applied and addition of *t*-butanol increases conversion by changing the equilibrium constant of activity coefficients. The novelty and importance of this work are, firstly, proving the feasibility and practicability of the strategy to explore efficient IL reaction systems by a COSMO-RS *a priori* screening from a large number of available structures, and theoretical evaluation on multiple interactions within the system, in combination with experimental validation. Secondly, we have shown that the *t*OMA·TFA–*t*-butanol system allows access to a variety of compounds with multiple OH groups (*e.g.* flavonoids and sugars) as an efficient system for enzymatic conversion (related investigations are in progress in our group), indicating the general value of this approach.

Experimental

Materials

L-Ascorbic acid (99%) was purchased from Sigma-Aldrich Co. (St Louis, USA). Conjugated linoleic acid (CLA), with >80% (9*Z*,11*E*)- and (10*E*,12*Z*)-isomer content, was purchased from Cognis Deutschland GmbH (Manheim, Germany). Methyltrioctylammonium trifluoroacetate (*t*OMA·TFA) of 99.7% purity was from Merck KGaA (Darmstadt, Germany). 1-Butyl-3-methylimidazolium trifluoromethanesulfonate ([BMIM][CF_3SO_3]) and all ionic liquids were procured from Solvent Innovation GmbH (Köln, Germany) and were of 98% minimum purity. Novozym 435 (from *Candida antarctica*) was a gift from Novozymes A/S (Bagsvaerd, Denmark).

Typical experimental procedure and analysis

In a typical reaction, 1.22 mmol ascorbic acid (0.2148 g) was dissolved in 0.5 mL IL, and then 0.5 mL of *t*-butanol, heptane or toluene was added, followed by the addition of 6.1 mmol CLA (1.714 g) for mixing. For the reaction in pure ILs, 1.22 mmol ascorbic acid was directly dissolved in 1 mL IL, followed by the addition of 6.1 mmol CLA. The reaction was conducted in a jacketed reactor at 70 °C with magnetic agitation at 500 rpm, 200 mg activated molecular sieves and 200 mg Novozym 435. The evolution of the reaction was monitored by periodic sample withdrawal and HPLC analysis after dissolving the samples in dimethyl sulfoxide (DMSO).

Examination on the effects of temperature on the reactions were performed under conditions identical to those above. Effects of substrate concentrations were also examined, based on the above reaction conditions with various concentrations of ascorbic acid. The CLA/AA ratio was kept at 5 : 1, and the volume of solvent (pure ILs or their mixture with molecular solvents) was also kept constant at 1 mL. Effects of the substrate ratio were evaluated by employing the above conditions with ascorbic acid fixed at 1.22 mmol and CLA varied from 1.22 to 12.2 mmol.

All reactions were performed in duplicate and the means of two determinations were used for result evaluation.

HPLC analysis of reaction mixtures was carried out on an Agilent HPLC system (1100 series, Agilent Technologies, Germany) with an Ascentis RP-C8 column (25 cm × 4.6 mm, 5 μm, Supelcosil Inc., Bellefonte, PA). This system was equipped with an autosampler, on-line degasser, column heater, and ultraviolet diode-array detector (UV-DAD). The detection wavelength was set at 254 nm. A 20 μL aliquot was dissolved in 1 mL DMSO, and the enzyme was removed by centrifugation. The binary mobile phases were methanol and triethylamine–acetic acid buffer (10 mM, pH 4.0). The elution gradient was as follows: start with 30% methanol and increase to 100% methanol over 10 min; hold for 7 min and then reduce to 30% methanol over 3 min; hold at this ratio for 10 min. The mobile phase flow rate was 1.0 mL min^{-1} and column oven temperature 35 °C.

Area percentages of ascorbic acid and CLA ascorbyl ester were used as weight for the mole conversion calculation based on mass balance. The volumetric productivity of CLA ascorbyl ester (g L^{-1} or mol L^{-1}) is defined as the amount of the product generated per unit volume. This was estimated based on the concentration of ascorbic acid and its conversion, and calibrated by the standard curves of ascorbyl palmitate.

Model processing

Generation of molecular COSMO files was implemented on Turbomole 5.8. Infinite dilution activity coefficients used for $K_{eq,\gamma}$ thermodynamic calculations and solubilities of ascorbic acid in ILs and *t*-butanol computation with a non-iterative mode were carried out on CosmothermX_2.2 (COSMOlogic GmbH & Co KG, Leverkusen, Germany).[6] Cavity radius (Å) used is the optimized data: C (2.00), H (1.30), O (1.72), N (1.83), S (2.16) and P (2.106).

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