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SHORT COMMUNICATION

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Chymotrypsin-Catalyzed Peptide Synthesis in Deep Eutectic Solvents

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Deep eutectic solvents (DESs) are formed by mixing quaternary ammonium salts (e.g., choline chloride) and hydrogen-bond donors (e.g., glycerol or urea), which leads to biodegradable and readily available ionic solvents at room temperature. Analogous to other ionic liquids, DESs represent a promising reaction media if hydrophobic and hydrophilic substrates need to be combined. This paper assesses DESs as reaction media for chymotrypsin-catalyzed peptide synthesis. After careful determination of the reaction conditions (e.g., water content, enzyme loading), α-chymotrypsin displayed high activity for peptide synthesis in choline chloride/glycerol mixtures to afford productivities of ca. 20 $g L^{-1} h^{-1}$ and with complete selectivity for the peptide, which is in contrast to the detrimental hydrolysis pathway observed in aqueous media. The nonimmobilized suspended enzyme could be reused several times by simple filtration with excellent to moderate activities. Overall, the results reported suggest that choline chloride based DESs may become promising neoteric solvents for peptide synthesis through biocatalysis.

Introduction

Over the last decades, enzyme catalysis has been established as a broadly applicable and useful technology for organic synthesis, in particular for regio-, enantio-, and chemoselective processes. Together with advances in molecular biology – for genetic design and further accessibility of biocatalysts on a large scale – an important reason for such increasing utility is the fact that many enzymes can operate under non-aqueous conditions, the so-called nonconventional media, which opens the possibility of combining high substrate loadings and biocatalysis with a clear focus on industrial synthesis.[1] After the seminal work developed by Klibanov, mostly with hydrolases in organic media,[2] in recent years other enzymes such as oxidoreductases and transaminases have been reported to display excellent activities and selectivities in non-aqueous media, including solvent-free systems or the use of different organic solvents.[3] Moreover, a broad number of ionic liquids (ILs) have also been successfully tested as useful (co)solvents for enzyme-catalyzed reactions. The possibility of dissolving challenging materials or substrates with different polarities has emerged as an added value of these neoteric solvents.[4] Yet, recently the environmental impact of commonly used ILs has been put under discussion.[5]

Besides their potential environmental impact, the cost associated with the synthesis and use of ILs has been typically a hurdle for their further practical use. In this respect, aiming to keep the versatility of the properties that ILs display, while at the same time providing more environmentally friendly and cost-effective options, deep eutectic solvents (DESs) have emerged as a novel generation of ionic solvents with promising applications.[6–9] DESs are formed by mixing quaternary ammonium salts (e.g., choline chloride as the most commonly used) with compounds capable of forming hydrogen bonds with the first component (e.g., amines, amides, alcohols, or carboxylic acids, the so-called HBD: hydrogen-bond donor). After mixing and upon gentle stirring and warming (up to 100 °C), the HBD disrupts the crystalline structure of the quaternary ammonium salt, which decreases the freezing point (T_f) of the mixture and generates (in some cases) liquids at room temperature. The classic example is the combination of choline chloride (m.p. 302 °C) with urea (m.p. 133 °C) in a 1:2 molar ratio to create a room-temperature liquid DES (T_f = 12 °C).[6–9] The synthesis of DESs is often straightforward, does not require further purification steps, and no byproducts are formed. As starting materials, biodegradable, readily available, and inexpensive compounds are often used, and thus, this route has a promising environmental prognosis.[9] Considering these aspects, research into the applications of DESs in different chemical segments has flourished over the last decade.[6–9] With regard to applications in biocatalysis, several enzyme-catalyzed processes with the use of DESs as solvents have been reported, and they typically focus on lipase-catalyzed (trans)esterifications and aminolysis reactions.[10] Actually, urea is a well-known denaturing agent for proteins, but lipases remain largely stable and active in choline chloride/urea (ChCl:Ur) DESs.[10]

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These potential advantages of DESs – biodegradability, ease of preparation, availability – together with their capability of dissolving a broad range of complex natural products and molecules[8] might make them appealing neoteric solvents for industrial reactions. An example of this might be represented by peptide synthesis, as peptides and oligopeptides are added-value products with a broad range of market-oriented applications.[11] In this respect, one of the challenges for peptide synthesis is the reaction medium, as hydrophobic and hydrophilic substrates typically need to be combined at the same time. Therein, the use of aqueous solutions is in many cases not recommended, as water often shifts the equilibrium towards hydrolysis rather than to peptide synthesis. For such purposes, non-aqueous ILs have been used as solvents for the production of peptides with the use of either chemo- or biocatalysts[12] such as proteases.[13] Moreover, another approach involving the use of proteases for peptide synthesis comprises the formation of eutectic mixtures of substrates (e.g., two amino acids or peptides) with the aid of adjuvants (e.g., small amounts of ethanol or water), which leads to "solvent-free" mixtures with high substrate loadings[14] and to "solid-to-solid" reactions.[15] In the field of choline chloride based DESs, however, proteases have been scarcely explored.[16] Addressing the potential advantages of DESs for biocatalytic industrial processes, in general, and the added value that they may bring to the arena of peptide synthesis, in particular, in this paper the use of proteases for peptide synthesis in choline chloride based DESs is explored.

Results and Discussion

Different choline chloride based DESs were prepared by gently mixing their components under mild conditions (up to 100 °C). As HBDs, several biomass-derived compounds were used, namely, glycerol, urea, xylitol, and isosorbide (Figure 1).

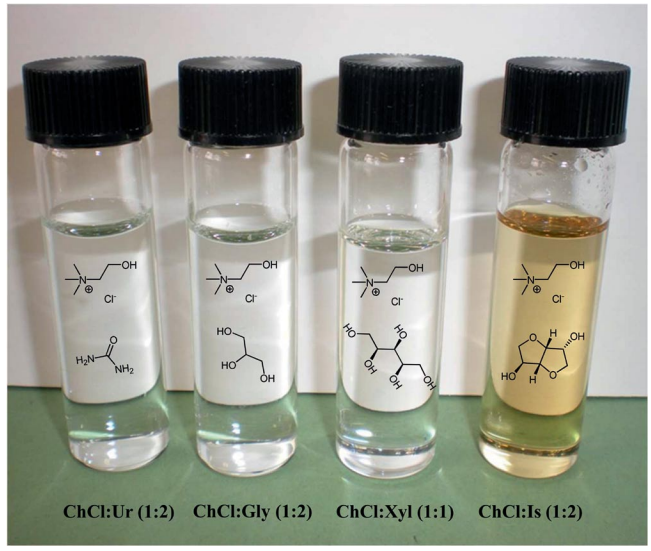
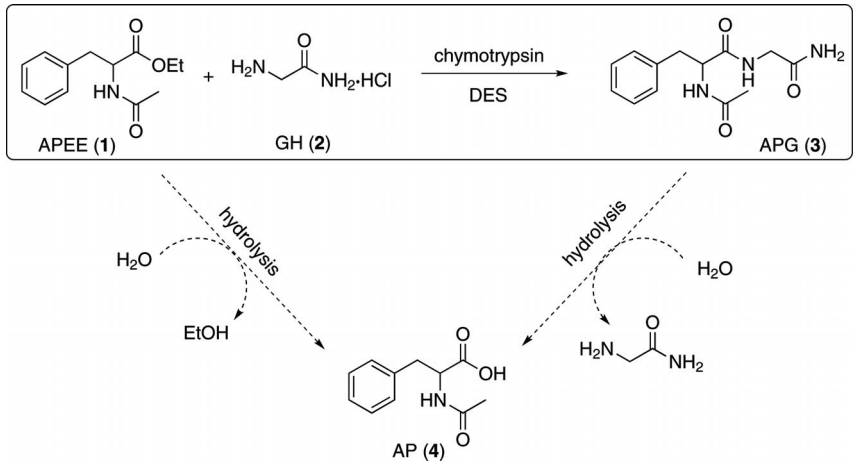


Figure 1. DESs employed in this study (see the Supporting Information for details).

To assess DESs in protease-catalyzed processes, α-chymotrypsin was used as the biocatalyst. As a prototypical reaction, the synthesis of the protected *N*-Ac-Phe-Gly-NH_2 peptide (APG, **3**) in different DESs was used, starting from *N*-acetylphenylalanine ethyl ester (APEE, **1**) and glycinamide hydrochloride (GH, **2**) as substrates (Scheme 1). The reason for this election was the preponderance of APEE (**1**) to undergo dissolution in hydrophobic solvents, whereas GH (**2**) tends to be more soluble in hydrophilic solvents.[17] The resulting dipeptide, APG (**3**), has also found some therapeutic uses.[17]

The formation of the desired APG (**3**) dipeptide may be hampered by detrimental hydrolysis to yield AP (**4**), especially if significant amounts of water are present.[15a,15c,15f,17] However, some molecules of water are crucial for the activity of enzymes in nonconventional media.[2,3] Thus, the reaction conditions must chosen carefully



Scheme 1. Chymotripsin-catalyzed model reaction used in this paper.

to minimize the hydrolytic side reaction, while at the same time enhancing the biocatalytic production of **3** and thus taking full benefit of the neoteric nonconventional media (e.g., ease of dissolution of substrates with different polarities). Addressing these aspects, in a first set of experiments the influence of water in the reaction media was assessed. As a prototypical DES, choline chloride/glycerol (ChCl:Gly, 1:2 molar ratio) was used.[7c] The results are depicted in Figure 2.

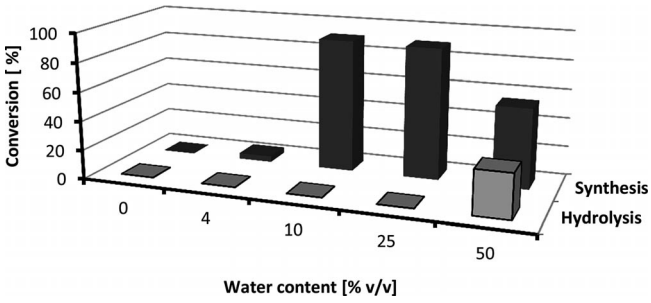


Figure 2. Effect of water (synthesis vs. hydrolysis) in α-chymotrypsin-catalyzed peptide synthesis by using *N*-acetylphenylalanine ethyl ester (APEE, **1**) and glycinamide hydrochloride (GH, **2**) as substrates. Reaction conditions: APEE (20 g L^{-1}), GH (10 g L^{-1}), α-chymotrypsin (4 mg mL^{-1}) ChCl:Gly DES [0–50% water (v/v)], room temperature, 24 h. Conversions determined by ^{1}H NMR spectroscopy.

As can be observed (Figure 2), lower amounts of water (up to 5% v/v) led to virtually no protease activity in ChCl:Gly media, which suggests denaturation of the enzyme under those extremely dry conditions. Remarkably, the addition of 10% water (v/v) enabled high enzymatic activity, and peptide **3** was obtained in 90% yield. Excellent results were also observed if up to 25% water (v/v) was added. In this range (10–25% v/v), the hydrolytic reaction was completely suppressed. At higher amounts of water (50% v/v), hydrolysis was significantly observed. Obviously, for practical considerations in peptide synthesis, a broad range of water contents (10–25% v/v) should be available to allow both excellent enzymatic activity and outstanding selectivity for the synthetic reaction. Looking beyond and envisaging this DES-based strategy as a useful concept for other more difficult-to-dissolve substrates (e.g., oligopeptides), 10% water (v/v) was taken in further experiments. It may be expected that a broad range of substrates may be dissolved in such DES/water mixtures (90–10% v/v), whereas at the same time excellent enzymatic operational conditions (stability and activity) may be achieved. Once the reaction was finished, the downstream processing for the DES was conducted upon the addition of water (to break the DES) and extraction with ethyl acetate.[18] Gentle warming of the DES/water mixture to remove the water led to the recovery of the DES, as reported previously.[7a,7b] Likewise, considering further practical uses of the DES, preliminary assessments in the literature suggest a promising diminishing corrosiveness of choline chloride based DESs with the use of non-acidic HBDs (e.g., polyols).[19]

Once these bench reaction conditions were fixed (ChCl:Gly, 10% water, v/v), the enzyme loading was subsequently assessed to obtain insight into the reaction rate of the synthetic process, and especially to gain more information on the relationship between synthesis and hydrolysis, and to determine the best reaction conditions for further optimizations of the reaction. The results for two different enzyme loadings (20 and 40 mg mL^{-1}) are depicted in Figure 3.

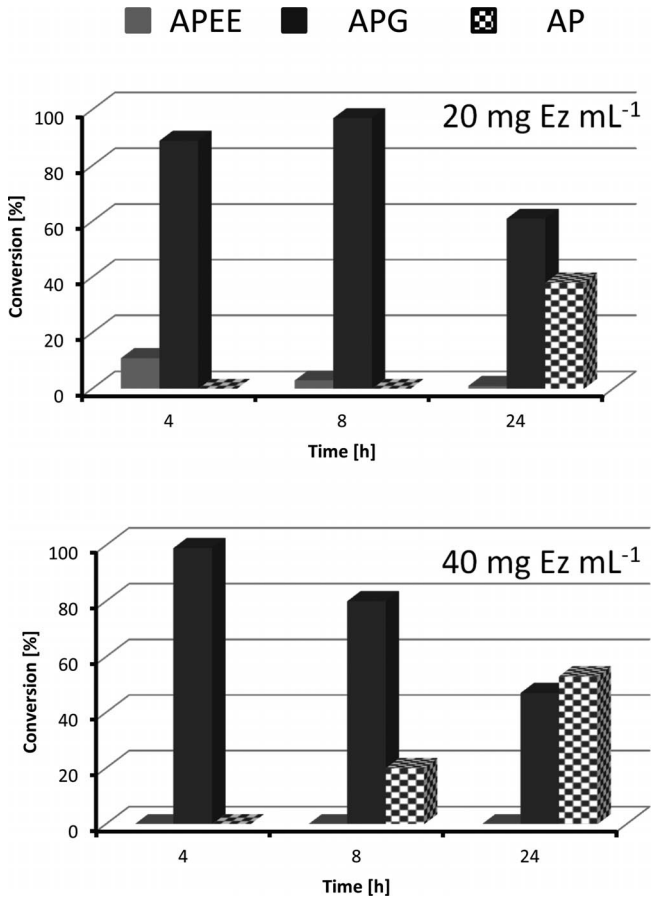


Figure 3. α-Chymotrypsin-catalyzed peptide synthesis by using *N*-acetylphenylalanine ethyl ester (APEE, **1**) and glycinamide hydrochloride (GH, **2**) as substrates. Reaction conditions: APEE (20 g L^{-1}), GH (10 g L^{-1}), ChCl:Gly DES [10% water (v/v)], room temperature with α-chymotrypsin at a loading of 20 mg mL^{-1} (top) and 40 mg mL^{-1} (bottom) Conversions determined by ^{1}H NMR spectroscopy.

The synthetic reaction (peptide-bond formation) proceeded fast with virtually full conversion and outstanding selectivities (for synthesis) at short reaction times (4 h), whereby no hydrolytic reactions were observed. As expected, significant amounts of hydrolysis product **4** were observed in both cases at longer reaction times (24 h), as well as at 8 h if higher enzyme loadings were used. Therefore, considering the importance of fast reactions with high selectivities, for practical purposes the assessed reaction may be performed in short reaction times (4 h) to afford

complete selectivity for desired peptide **3**. Under these controlled conditions [water amount (10% v/v) and enzyme loading (40 mg mL^{-1})], full conversion with excellent selectivity for APG (**3**) were achieved.

In the next set of experiments, the substrate loading was assessed. As a starting point, for the above-described reaction conditions, a substrate loadings of APEE (**1**) of ca. 20 g L^{-1} was applied, which led to full conversion in 4 h. Envisaging industrial conditions for peptide synthesis and taking advantage of the capability of DESs to dissolve these challenging substrates, the loading of APEE (**1**) was significantly increased up to 170 g L^{-1}. The results are depicted in Figure 4.

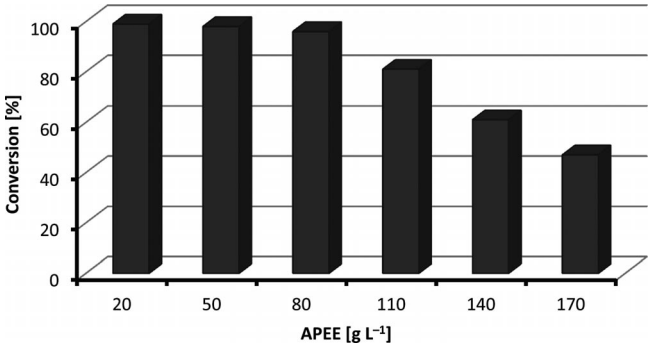


Figure 4. Peptide-catalyzed reactions at higher substrate loadings. Reaction conditions: APEE (20–170 g L^{-1}), GH (10–81 g L^{-1}), α-chymotrypsin (40 mg mL^{-1}), ChCl:Gly DES [10% water (v/v)], room temperature, 4 h. Conversions determined by ^{1}H NMR spectroscopy.

Gratifyingly, the enzymatic process in the DES was revealed to be quite robust, and thus, substrate loadings in the range of 80 g L^{-1} maintained full conversion and perfect synthetic selectivities under the (still) unoptimized conditions. At higher substrate loadings, lower conversions were gradually observed (at 4 h reaction time). By considering these data together with the observations reported in Figure 3, it may be envisaged that a reaction time between 4 and 8 h should still allow room to enhance peptide production without compromising the selectivity through the expected concomitant hydrolysis route. In any case, the results clearly support the idea that DESs may be useful reaction media for proteases under industrially oriented conditions (high substrate loadings and conversions, short reaction times, etc.), and thus, productivities of ca. 20 g L^{-1} h^{-1} with excellent selectivities are observed.

The reactions were performed by using free, nonimmobilized α-chymotrypsin that remained suspended as a powder in the DES media. With the goal of assessing whether reuse of the enzyme was feasible, the suspended powder was recovered upon filtration, washed with 2-propanol, and directly used in the next catalytic cycle (see the Exp. Section for details). The results are depicted in Figure 5.

As observed, the nonimmobilized suspended enzyme could be directly used over 3–4 cycles while maintaining excellent to moderate activities in the model reaction studied, with complete selectivity for the synthesis of the peptide (vs. hydrolysis) in all cases. After those reaction cycles, clear deactivation of the biocatalyst was observed. Taking into account that in this case the enzyme was not immobilized, it can be expected that a more robust immobilized system may lead to more stable catalysts for the reaction proposed. In any case, the free enzyme already shows promising stability in DESs.

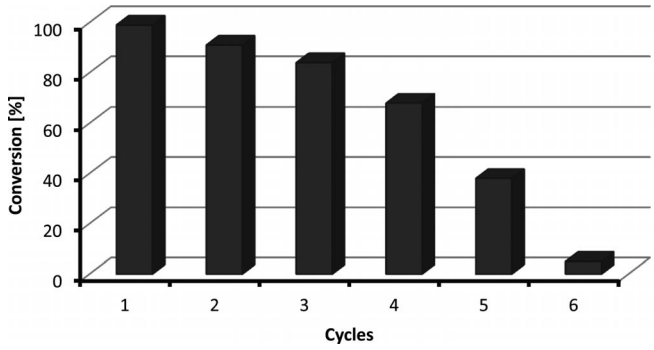


Figure 5. Enzyme recycling. Reaction conditions: APEE (20 g L^{-1}), GH (10 g L^{-1}), α-chymotrypsin (20 mg mL^{-1}), ChCl:Gly DES [10% water (v/v)], room temperature, 2 h. Conversions determined by ^{1}H NMR spectroscopy.

Finally, different choline chloride based DESs were assessed for the protease-catalyzed reaction. The rationale behind these studies was the assumption that proteases—for industrial purposes—may be used for a broad range of diverse substrates, including (protected) amino acids, oligopeptides, and so on. Some of these substrates might dissolve well in some DESs, whereas other substrates might perform better in different DESs (or in mixtures of them). Thus, the preparation of several DESs with positive enzymatic activities might be an asset. Herein, four choline chloride based DESs (Figure 1) with glycerol (ChCl:Gly, 1:2), urea (ChCl:Ur, 1:2), isosorbide (ChCl:Is, 1:2), and xylitol (ChCl:Xyl, 1:1) as HBDs were assessed. The results are depicted in Figure 6.

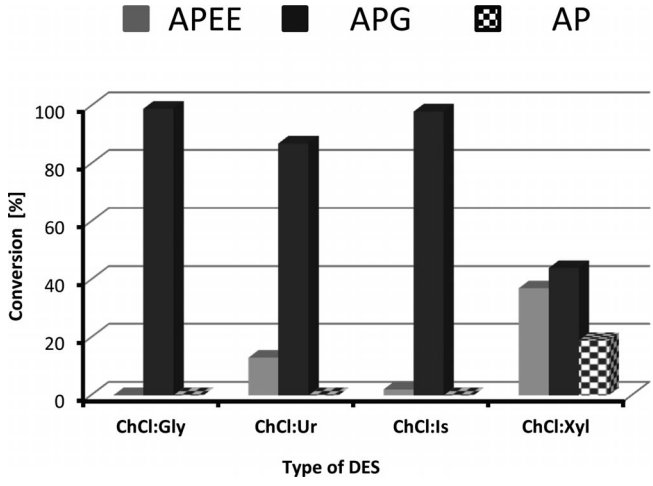


Figure 6. α-Chymotrypsin-catalyzed peptide synthesis by using *N*-acetylphenylalanine ethyl ester (APEE, **1**) and glycinamide hydrochloride (GH, **2**) in four different DESs. Reaction conditions: APEE (20 g L^{-1}), GH (10 g L^{-1}), α-chymotrypsin (20 mg mL^{-1}), DES [10% water (v/v)], room temperature, 4 h. Conversions determined by ^{1}H NMR spectroscopy.

As can be observed, different DESs led to positive results in the protease-catalyzed synthesis. When glycerol or isosorbide were used as the HBD, virtually full conversion was reached, with complete selectivity for synthetic product **3**. Moreover, despite the denaturing effect of urea, ChCl:Ur provided high conversions as well. Interestingly, the use of a DES composed of choline chloride and xylitol provided much lower conversion to the ester, together with significant formation of the hydrolysis product.

Conclusions

In summary, chymotrypsin has been successfully assessed in choline chloride based DESs for the synthesis of peptides. DESs are biodegradable and can be straightforwardly produced; at the same time, they enable the dissolution of amino acids and peptides, which can open interesting options for industrial processes. Under the reported conditions in a prototypical reaction, productivities of ca. 20 $g L^{-1} h^{-1}$ were reached with completely controlled and diminished hydrolysis. Furthermore, the suspended nonimmobilized free enzyme could be reused over several cycles before deactivation. The determination of proper enzyme immobilization strategies might improve the recycling significantly. The results demonstrate the successful application of choline chloride based DESs to enzymatic processes of significant industrial importance, which further substantiates the synthetic and practical potential of these reaction media for practical applications under the sustainable chemistry principles.

Supporting Information (see footnote on the first page of this article): Experimental procedures and copies of the NMR spectra of the solvents (DESs), reagents, and products

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