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Ionic Liquids and Proteases: A Clean Alliance for Semisynthesis

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Herein we present the first report on protease-catalysed ligation of cleavage-sensitive peptide and protein fragments in ionic-liquid-containing solvent systems. By applying the newly established [MMIM][Me_2PO_4]/buffer mixture as a reaction medium, significant advantages over purely aqueous or conventional organic solvent-containing media could be identified, including in particular the use of active wild-type proteases as biocatalysts, the suppression of any competitive proteolytic side reactions, the high turnover rates compared to classical organic solvents and the high stability of chemically labile reactants.

Introduction

The chemoselective ligation of peptide fragments has been established as a powerful tool for the synthesis of functional biological macromolecules, including synthetically engineered polypeptides up to proteins.[1] Generally, the ligation approaches that have been developed can be distinguished into two basic strategies, that is, the coupling of either 1) fully protected synthetic peptides or 2) unprotected functionalised fragments derived from chemical or recombinant techniques. Examples for the latter involve the well-known native chemical ligation technology that mediates the selective condensation of an N-terminal peptide α-thioester with a C-terminal cysteine-containing peptide,[2] the Staudinger ligation, which is based on the specific reaction between a phosphinothioester and peptide azide[3] and the protease ligation method in which specific peptide esters, especially substrate mimetics, react with a broad variety of nucleophilic peptide species in a regio- and stereospecific manner.[4]

Although the practicability of these strategies regarding the increasing size of peptidic targets is beyond any doubt, the synthesis of more complex, therapeutically relevant membrane proteins is seriously handicapped mainly by their poor solubility in aqueous solvents or limited stability in the organic–aqueous media that is required for purification, and in particular, subsequent ligation.

Successful synthesis of those difficult sequences therefore demands novel powerful solvent systems with tuneable solubility properties, compatibility to the labile structure of proteins as well as chemical and enzymatic ligation approaches; this complex constellation of needs might be fulfilled by using ionic liquids. This relatively new class of nearly nonvolatile, purely ionic materials offers huge potential to optimise reaction processes by its adaptable, diverse and unique set of physicochemical properties that lead evidently to remarkable improvements compared to conventional solvent systems.[5] Whereas the use of ionic liquids as solvent in the field of organic chemistry is increasingly documented and booming constantly, with respect to biocatalysis a comparably small number of oxidoreductase and hydrolase-catalysed reactions has been published to date.[6] Moreover, only two reports that deal with the thermolysin and chymotrypsin-catalysed conversion of single amino acid reactants without any competing cleavage sites[7] clearly make enzymatic polypeptide or even protein ligation in ionic liquids a vast and innovative challenge.

Herein we report on the first successful ligation of cleavage-sensitive peptide and protein fragments by the use of ionic liquids as a reaction medium additive. By retaining the activity and selectivity of wild-type proteases, coupling reactions strongly benefit from both the excellent solubility and stability of the reactants in the cosolvent, and in particular, from the complete suppression of proteolytic side reactions, which are the main drawbacks when native proteases are used as biocatalysts for peptide and protein ligation.

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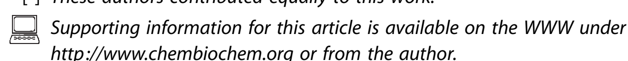
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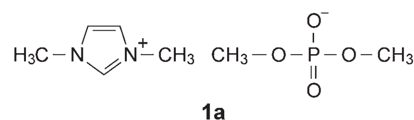
Results and Discussion

In our initial studies we have proven the general scope of ionic liquids with respect to maintaining protease activity, solubility of polypeptides and water miscibility by using an individually selected set of ionic liquids that are composed of cations such as differently substituted imidazolium, quarternary ammonium, and phosphonium and anions, including triflate, acetate, thiocyanate, dimethylphosphate, tosylate, chloride and methylsulfate (Table S1 in the Supporting Information). Thus, water-immiscible ionic liquids and also those that are already known for their instability (for example, tetrafluoroborates) were consciously excluded. With the remaining ionic liquids selected model reactions have been performed by using three proteases with distinct specificities, that is, the Tyr-, Arg- and Glu-specific chymotrypsin, trypsin and V8 protease, respectively, as biocatalysts. To evaluate the individual effects of each ionic liquid on both the proteases' activities and solubility of hydrophobic polypeptides, the screening reactions were performed in a kinetically resolved manner at different ionic liquid and reactant concentrations. Whereas variations of the ionic liquid content directly monitor the effect of the additive on the proteases' activity, variations of the latter allow simultaneously for conclusions to the general solubility behaviour of the distinct ionic liquid towards increasing concentrations of the respective low-water-soluble peptides. To meet the latter requirement, the rather hydrophobic peptide LIVNAVLQPVAAGAY was selected as the model acyl acceptor component. The peptide itself was used in concentrations of 1 mM, at which it is still soluble in the conventional aqueous system, as well as in ten-fold higher concentrations, which lead to peptide precipitation under similar conditions. As acyl donors, simple ethyl esters of the general structure Bz-Xaa-OEt (1 mM) with Xaa being adapted to the individual primary specificity of each enzyme (Arg for trypsin, Tyr for chymotrypsin and Glu for V8 protease) were selected. The ionic liquids themselves were used in concentrations of 50, 60, and 70% (*v*/*v*) as additives to a buffered aqueous solvent system. Temperature and pH optima were adjusted according to the individual requirements of the used enzymes.[8] Due to the limited water solubility of the acceptor peptide, the comparative reactions without ionic liquid were restricted to those that used 1 mM acceptor peptide. Even in this case, however, 5% DMF had to be added to ensure the complete solubility of all reactants. To control for non-enzyme-related hydrolysis and aminolysis of the ester moieties that might interfere with the enzymatic syntheses, reactions without enzyme were performed for each single ionic liquid system in parallel, which gave no indication of any spontaneous ester or peptide conversions regardless of the presence or absence of the respective ionic liquid. Considerably more diffuse results were found for the reaction systems that contained both the respective enzyme and ionic liquid (Table S1). The effects that were observed ranged from a complete loss of enzyme activity without any conversion of the reactants, as was found for all tosylates, chlorides and sulfates, over an exclusive hydrolysis of the acyl donor esters without any significant formation of the desired ligation products as it could be observed in the ammonium-derived ionic liquid system, to the promotion of enzyme-catalysed peptide bond formation between the respective reactants, which was found for the alkyl imidazolium acetate, triflate, thiocyanate and dimethylphosphate. Interestingly, these general effects were found to be independent of the individual enzyme and became already evident at the lowest ionic liquid concentration used. Moreover, the ionic liquids that support peptide bond formation also show similar quantitative trends for all three enzymes with respect to their catalytic activity and synthesis efficiency. Regarding both parameters, 1,3-dimethylimidazolium dimethylphosphate ([MMIM][Me_2PO_4], **1a**) was



identified as the most promising ionic liquid because it mediates not only the highest reaction rates and peptide product yields for all three enzymes, but also ensures the complete solubility of all reactants.[9]

Next, we evaluated the general suitability of the selected [MMIM][Me_2PO_4] as a reaction medium additive for enzymatic ligation reactions of cleavage-sensitive peptide reactants. For this purpose, three pentapeptides of the general structure Leu-Ala-*Xaa*-Ala-Gly were used; the *Xaa*-Ala motif is an endogenous cleavage site for each enzyme and was used as the acyl acceptors. Because of the significantly higher synthetic value of substrate mimetics over conventional acyl donors, we further exchanged the simple ethyl esters with Bz-Gly-OGp (trypsin and chymotrypsin) and Bz-Gly-SCm (V8 protease), respectively.[4,10] Both the 4-guanidinophenyl ester (OGp) and carboxymethyl thioester (SCm) moieties are well known to mediate the acceptance of nonspecific acyl residues by these enzymes, and thus allow for sequence-independent and therefore highly flexible peptide bond syntheses that are infeasible with conventional acyl donor components.[4,8,10] With exception of the reactant concentrations, which were fixed at 2 mM (acyl donor) and 20 mM (acyl acceptor), the reactions themselves were performed under similar conditions as described above. Similarly, parallel control reactions without enzyme were performed, which in all cases and independent of the presence of the ionic liquid gave again no hints of any spontaneous reaction of the reactants, including the exclusion of nonenzyme-catalysed (for example, ionic liquid) peptide bond formations. A completely different picture was found for the enzyme-containing reaction systems. Generally, a comparison of the ligation reactions under purely aqueous conditions with that by using [MMIM][Me_2PO_4] as a cosolvent reveals a radical shift in enzyme preferences (Table 1). Whereas reactions without the ionic liquid either completely failed in terms of the formation of the desired full-length peptide product (V8 protease), or gave yields of temporarily accumulated intact peptide products in the range of only 19 to 45% (trypsin and chymotrypsin, respectively), the addition of [MMIM][Me_2PO_4] efficiently suppressed the undesired proteolytic cleavage of the respective

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Table 1. Influence of [MMIM][Me_2PO_4] on substrate-mimetics-mediated protease couplings of cleavage sensitive peptides.[a]

Protease/ acyl donor	Acyl acceptor	Yield [%] [MMIM][Me_2PO_4]/MOPS buffer (*v/v*) 0:100[b]	50:50	60:40	70:30
Chymotrypsin					
Bz-Gly-OGp	LA***Y***AG	45.1 (36.1)	59.6 (23.1)	86.0	87.4
Trypsin					
Bz-Gly-OGp	LA***R***AG	18.9 (50.3)	52.4 (19.9)	76.7	79.7
V8-protease					
Bz-Gly-SCm	LA***E***AG	0 (68.4)	52.5 (18.4)	76.5	78.9

[a] Enzyme-specific amino acid moieties within the peptide reactants are marked. Parentheses indicate competitive proteolysis of the respective specific peptide bond during synthesis, that is, the parenthetical data are the yields of the undesired proteolytically cleaved products, whereas the data outside the bracket give the yields of the appropriate intact (uncleaved) peptide products. [b] 5% DMF (*v/v*). Conditions: 25 °C (37 °C in the case of V8 protease), pH 8.0, [acyl donor] = 2 mM, [acyl acceptor] = 10 mM, aqueous system: [chymotrypsin] = 1.5×10^{-6} M, [trypsin] = 1×10^{-6} M, [V8 protease] = 1×10^{-5} M, ionic liquid system: [chymotrypsin] = 0.5×10^{-6} M, [trypsin] = 0.25×10^{-6} M, [V8 protease] = 0.3×10^{-5} M. Errors are less than 5% (±2.5%).

enzyme-specific peptide bonds in a concentration-dependent manner. In fact, whereas at equal volumes of [MMIM][Me_2PO_4] and buffer the proteases' cleavage activities are already significantly reduced but still remain detectable, the addition of 60% of the ionic liquid to the reaction medium causes a complete suppression of any undesired proteolyses of cleavage-sensitive peptide bonds. As a result, about 77 to 86% long-time stable peptide products were obtained in the 60% ionic-liquid-containing reaction mixtures (Figure 1). A further increase of the [MMIM][Me_2PO_4] concentration in the solvent system of up to 70% led to further, although only less pronounced, improvements in the yields of ligation; this indicates that the ionic liquid not only suppresses the proteolytic hydrolysis, but also decreases the competing hydrolysis activity of the proteases toward the acyl donor esters to some extent (Table 1). Like the effect on proteolysis, the latter shows a clear concentration dependency on the ionic liquid, but does not lead to a complete suppression of undesired hydrolysis of acyl donor esters. A quantification of both the proteases' proteolytic and hydrolytic activities as a function of the ionic liquid concentration is given in Figure 2. Accordingly, the two activities decrease with increasing amounts of [MMIM][Me_2PO_4] in a nonlinear manner, whereas this effect is much more pronounced for the proteolytic activity of the enzymes. The rationale behind the general reduction of both activities might be correlated to the lower concentration of water as a result of increasing amounts of ionic liquid. This hypothesis is supported by the fact that for both the proteolytic and hydrolytic reaction, water acts in the same way as an essential reaction partner. Therefore, lowering the water concentration should go along with a respective reduction in the rates of the two water-dependent reactions. This simple hypothesis alone does not explain, however, the

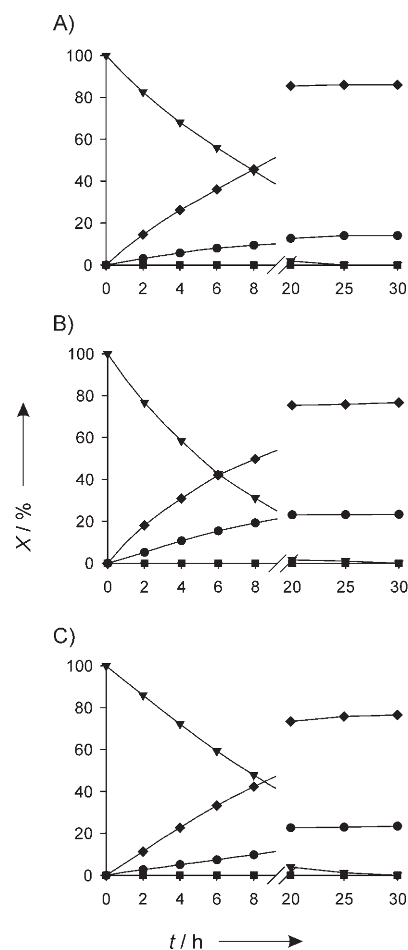

Figure 1. Course of the chymotrypsin, trypsin, and V8 protease-catalysed coupling of substrate mimetics with specific amino-acid-containing peptides in a 60:40 (*v/v*) [MMIM][Me_2PO_4]/buffer system: A) Bz-Gly-OGp and H-Leu-Ala-Tyr-Ala-Gly-OH with chymotrypsin; B) Bz-Gly-OGp and H-Leu-Ala-Arg-Ala-Gly-OH with trypsin; C) Bz-Gly-SCm and H-Leu-Ala-Glu-Ala-Gly-OH with V8 protease. (–▼–) Bz-Gly-OGp/SCm, (–●–) Bz-Gly-OH, (–◆–) Bz-Gly-Leu-Ala-(Tyr/Arg/Glu)-Ala-Gly-OH, (–●–) Bz-Gly-Leu-Ala-(Tyr/Arg/Glu)-OH. Conditions: 25 °C (37 °C in the case of V8 protease), pH 8.0, [acyl donor] = 2 mM, [acyl acceptor] = 10 mM, [chymotrypsin] = 0.5×10^{-6} M, [trypsin] = 0.25×10^{-6} M, [V8 protease] = 0.3×10^{-5} M, *X* = product yield. Errors are less than 5% (±2.5%).

significant differences in the degree of that reduction that is found for the proteolysis and hydrolysis rates of the enzymes. Thus, additional and more direct-acting effects of the ionic liquid can be expected to be important, in particular for affecting the proteases' proteolytic activity. Generally, in a large number of studies it has been already shown that catalytically relevant changes in the structure of proteases have a significantly higher impact on their proteolytic rather than their hydrolytic activity.[4] The higher demand of proteolytic reactions, based on chemically more stable peptide bonds, on the structural integrity of the enzyme compared to analogous hydrolysis reactions of more activated ester derivatives is discussed as the molecular reason for this phenomena. With respect to ionic liquids, such changes in the spatial structure of enzymes should be related to partial denaturation or unfolding effects. However, our attempts at directly measuring those ionic liquid-mediated structural changes by using routinely applied tech-

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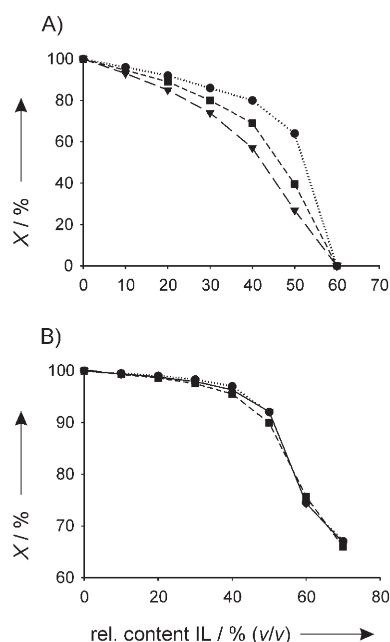

Figure 2. Effect of increasing [MMIM][Me_2PO_4] concentrations on the proteolytic and hydrolytic activity of chymotrypsin, trypsin, and V8 protease. A) Proteolytic activity was measured by using H-Leu-Ala-*Xaa*-Ala-Gly-OH (*Xaa* = Tyr: chymotrypsin; *Xaa* = Arg: trypsin; *Xaa* = Glu: V8 protease) as substrates. B) Hydrolytic activity was determined by using Bz-Gly-*O/SR* (*OR* = OGp: chymotrypsin and trypsin; *SR* = SCm: V8 protease) as substrates. (–●–) chymotrypsin, (–●–) trypsin, and (–▼–) V8 protease. Conditions: 25 °C (37 °C in the case of V8 protease), pH 8.0, [substrates] = 2 mм, [chymotrypsin] = 0.5×10^{-6} м, [trypsin] = 0.25×10^{-6} м, [V8 protease] = 0.3×10^{-5} м, *X* = relative reaction rate. Errors are less than 5 % (± 2.5 %).

niques such as CD or fluorescence spectroscopy completely failed due to serious interference by the ionic liquid. The same holds true for our NMR spectroscopic measurements, which we performed with shorter model peptides. In spite of these serious difficulties, a first experimental proof of a general impact of ionic liquids on the structure of proteins was obtained by measuring the influence of [MMIM][Me_2PO_4] on the *cis/trans* ratio of the Xaa–Pro bonds. By performing solvent-jump experiments[11] with peptides of the general structure Suc-Ala-Xaa-Pro-Phe-pNA (Xaa: Lys Glu, Phe), it could be shown that [MMIM][Me_2PO_4] affects the native *cis/trans* equilibrium in a significant manner. Quantitative analyses of this effect have further shown that the ionic liquid stabilises the *trans* isomer with remarkably high $\Delta\Delta G$ values between 2.7 and 3.8 kJ mol^{-1}, depending on the nature of the individual Xaa moiety (detailed results will be reported elsewhere). By taking into account that the aforementioned ΔΔG-values are related to the conformational impact of [MMIM][Me_2PO_4] on only one single peptide bond, much larger effects can be expected on the whole structure of proteins. Such structural changes could be finally responsible for the complete loss of the proteases' proteolytic activity already at relatively low ionic liquid concentrations, and thus rather high water concentrations at which the hydrolysis and aminolysis reactions that are based on more reactive ester substrates still occur. Generally it must be noted that the positive effects of [MMIM][Me_2PO_4] on the course of syntheses are paralleled by a reduction in the overall reaction rates. Unlike conventional organic solvent systems, [MMIM][Me_2PO_4] does not disturb the synthesis activity of proteases by five orders of magnitude,[12] but decreases the rate of product formation only by a factor of 100 to 1000 at the ionic liquid concentration that is related to a complete suppression of undesired proteolysis. Owing to the significant differences of the remaining water activities in aqueous ionic liquids[13] and classical water–organic solvent mixtures,[14] retained protease activities might be correlated to the higher water activity in the ionic liquid system. From a purely synthetic point of view, the lower rates of reaction in ionic liquids can be easily compensated for by increasing the enzyme concentration and/or extending of the incubation time. Undesired spontaneous reactions such as undesired acyl donor ester hydrolysis that was caused by longer reaction times might not be expected due to the high chemical stability that is found for the reactants under these conditions.

Finally, we evaluated the general suitability of ionic liquids for protease-mediated ligations of elongated peptide fragments by using the optimised 60:40 (*v/v*) [MMIM][Me_2PO_4]/buffer system. As a synthesis target we selected the truncated (46–92) sequence of the peptidyl prolyl *cis/trans* isomerase (PPIase) parvulin 10 from *E. coli* (Par10; Figure 3). Due to the

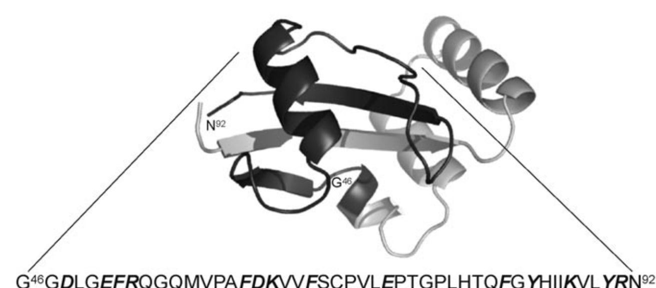

Figure 3. Sequence of the peptidyl prolyl *cis/trans* isomerase parvulin 10 (46–92) from *E. coli* and its 3D-structure in the full-length protein.[16] The potential cleavage sites of chymotrypsin (Tyr/Phe-Xaa), trypsin (Arg/Lys-Xaa) and V8 protease Glu/Asp-Xaa are marked.

primary catalytic function of PPIases to facilitate *cis/trans* isomerisations of Xaa-Pro bonds, they were found to be involved in the folding of newly synthesised proteins in the function of cell cycle control and in the immune system in the case of higher developed organisms.[15]

According to Figure 4 A, the synthetic route to Par10 (46–92) follows a single-step enzymatic coupling of an OGp or SCm ester of Par10 (46–62) and the 30-mer Par10 (63–92) fragment. As revealed by CD spectroscopy, the latter, although truncated, adopts a native-like structure as found in the full-length protein with respective α-helical and β-sheet regions (Figure 3). Thus, the site of ligation can be assumed to be an α-helical motif, which is known to be poorly recognised by proteases; in general, proteases prefer loop or β-sheet regions as the site of catalysis. This, together with the only low solubility of the fragment in purely aqueous media makes Par10 (46–92) a difficult sequence because it exhibits basic similarities to those of

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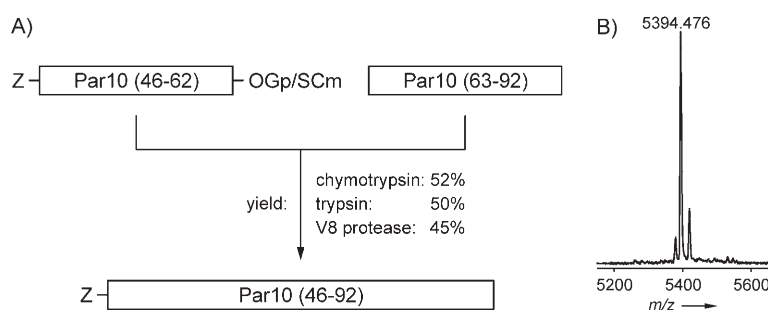

Figure 4. General course A) of the protease-catalysed coupling of Z-GGDLGEFRQGQMVPAFD-OGp/SCm (Par10 (46–62); OGp: chymotrypsin and trypsin; SCm: V8 protease) with KVVFSCPVLEPTGPLHTQFGYHIIKVLYRN (Par10 (63–92)) in a reaction medium that was composed of 60% (*v*/*v*) [MMIM][Me_2PO_4] and 40% (*v*/*v*) MOPS buffer, pH 8.0. B) Analysis of the chymotrypsin-catalysed synthesis of Z-Par10 (46–92) by MALDI-ToF mass spectrometry (calcd: 5392.71). Concentrations: [acyl donor] = 1 mm, [acyl acceptor] = 1 mm, [chymotrypsin] = 0.5×10^{-6} м, [trypsin] = 0.25×10^{-6} м, [V8 protease] = 0.3×10^{-5} м. Errors of the product yields reported are less than 5% (±2.5%).

membrane-associated proteins. The preceding chemical synthesis of the starting fragments itself was achieved by standard solid-phase Fmoc chemistry by using either Wang resin[17] for the preparation of Par10 (63–92) or Kenner's 4-sulfamylbutyryl aminomethyl safety catch resin[18] for synthesising the N-terminal Par10 (46–62) esters. Peptide release, deprotection, and purification led to the desired fragments, which were subsequently used for synthesis-economy reasons in equimolar concentrations of 1 mм for protease-catalysed ligations. Due to the excellent solvent properties of [MMIM][Me_2PO_4], no further additives were required to ensure complete solubility of all reactants. In the absence of the ionic liquid, significant volumes of at least 30% DMF had to be added to the reaction mixtures to compensate for the virtual dissolution of the fragments. The course of the synthesis reactions was finally analysed via HPLC and mass spectrometry either kinetically by applying an exact time control (reactions without ionic liquid) or simply after 20 h and complete ester consumption (reactions with ionic liquid). As was already found for the initial model reactions by using Bz-Gly-OGp/SCm, a strong preference of all three ionic-liquid-affected enzymes for catalysing the ligation reaction became evident. In fact, in the newly established [MMIM][Me_2PO_4]/buffer system besides the hydrolysis product of the heptadecapeptide esters, with chymotrypsin (52%), trypsin (50%) and V8 protease, 45% of the expected Par10 (46–92) were formed in spite of the difficult sequence and nonpreferred structure of the starting Par10 (63–92) fragment (Figure 4B). Truncated peptide products due to undesired proteolytic cleavages after enzyme specific amino acid moieties could not be detected. A completely opposite behaviour was found however, in the control reactions under conventional conditions without ionic liquid, in that competitive cleavages of the peptide reactants were favoured regardless of the protease used. Interestingly, even if applying an exact time control on the synthesis reactions that lacked [MMIM][Me_2PO_4], the desired intact ligation product could not be obtained at all; this proves the excellent behaviour of ionic liquids as cleavage-suppressing additives in protease-mediated ligation reactions. Initial up-scaling studies further demonstrated the power of [MMIM][Me_2PO_4] as an efficient solvent system. In fact, reactions with even tenfold higher reactant concentrations resulted in similar efficiencies and gave no hints of any reactant or product precipitation.

Conclusions

In summary, we present the first report on protease-catalysed ligation of cleavage-sensitive peptide and protein fragments in ionic-liquid-containing solvent systems. By applying the newly established [MMIM][Me_2PO_4]/buffer mixture as a reaction medium, significant advantages over purely aqueous or conventional organic solvent-containing media could be identified, including in particular the use of active wild-type proteases as biocatalysts, the suppression of any competitive proteolytic side reactions, the high turnover rates compared to classical organic solvents and the high stability of chemically labile reactants. These characteristics together with the powerful and tuneable solubility properties strongly suggest the enormous potential of ionic liquids for enzymatic peptide synthesis in general, and in particular for the ligation of difficult sequences with low water solubility that are becoming increasingly important, especially in terms of membrane-associated proteins. Moreover, because nonenzymatic ligation strategies suffer from restricted solubilities and stabilities of the peptide reactants or protein products, one can expect that ionic liquids should be equally qualified as solvent/cosolvent for purely chemical ligation approaches. Studies in this direction are presently under way.

Experimental Section

Materials: TPCK-treated bovine trypsin (EC 3.4.21.4), TLCK-treated bovine chymotrypsin (EC 3.4.21.1), V8 protease (EC 3.4.21.19), and the Bz-Arg-OEt and Bz-Tyr-OEt esters were obtained from Fluka, Sigma or Bachem. Proteases and ester derivatives were used without further purification. All ionic liquids were obtained from Solvent Innovation (Köln, Germany) or Fluka and exhibited an accredited purity of higher than 99.5% (Solvent Innovation: 1-Butyl-3-methyl-imidazolium 2(2-methoxyethoxy)ethylsulfate, 1-Ethyl-3-methyl-imidazolium tosylate, 1,3-Dimethyl-imidazolium dimethylphosphate, 1-Methyl-3-octyl-imidazolium chloride, and 2-Hydroxyethyl-trimethyl-ammonium dimethylphosphate) and 97% (Fluka: 1-Butyl-3-methyl-imidazolium acetate, 1-Butyl-3-methyl-imidazolium triflate, 1-Butyl-3-methyl-imidazolium thiocyanate, 1,3-Dimethyl-imidazolium methylsulfate, and Tetra(2-methylpropyl)methyl-phosphonium tosylate), respectively. Because it is known that even minor impurities in ionic liquids can affect their solvent properties,[5d,6d] each ionic liquid was further analysed individually by HPLC and ^{1}H NMR spectroscopic studies. If the ionic liquid contained a phosphorus atom, additional ^{31}P NMR spectroscopic analyses were performed. ^{1}H and ^{31}P NMR spectroscopic measurements were adjusted to ensure purities of even higher than 99.9%. Only ionic liquid charges that gave no detectable interfering signals from respective

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impurities in that range were used in this work. All further substances, including the materials for the synthesis of peptides, amino acid and peptide esters were products of Bachem, Fluka, Merck, Aldrich or Novabiochem. Unless otherwise stated, all reagents were of the highest commercial purity available.

Chemical syntheses: The amino acid ethyl ester Bz-Glu-OEt was synthesised by esterification of Boc-Glu(O*t*Bu)-OH with an excess of EtOH by using a *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate/*i*Pr_2EtN activation protocol. TFA treatment of Boc-Glu(O*t*Bu)-OEt resulted in the respective deprotected ester derivative that was finally benzoylated by using activated benzoic acid. The amino acid carboxymethyl thioesters Bz-Gly-SCm and Boc-Asp-(O*t*Bu)-SCm were synthesised by coupling of Bz-Gly-OH and Boc-Asp(O*t*Bu)-OH with thioglycolic acid by using the mixed anhydride method (isobutylchloroformiate/NEM). Amino acid 4-guanidinophenyl esters were prepared by direct condensation of the N^{α}-protected amino acid with 4-[*N*′,*N*′′-bis(Boc)guanidino]-phenol by using a TBTU/ iPr_2EtN activation protocol. Catalytic hydrogenation over Pd of Z-Asp(O*t*Bu)-OGp(Boc,Boc) and TFA treatment of Bz-Gly-OGp(Boc,Boc) and Boc-Asp(O*t*Bu)-SCm resulted in the respective deprotected ester derivatives. The peptides LAEAG, LARAG, LAYAG, LIVNAVLQPVAAGAY, and the Par10 (63–92) KVVFSCPVLEPTGPLH TQFGYHIIKVLYRN were prepared with a fully automated peptide synthesizer (Applied Biosystems) by using standard Fmoc chemistry and Wang resin,[17] respectively. After simultaneous peptide release and side-chain deprotection, the peptides were precipitated with dry diethyl ether and purified by preparative HPLC. The esters Z-GGDLGEFRQGQMVPAFD-OGp and Z-GGDLGEFRQGQMVPA FD-SCm were synthesised by using the alkanesulfonamide safety catch linker.[18] The first amino acid was loaded onto 4-sulfamylbutyryl aminomethyl resin by one benzotriazole-1-yl-oxy trispyrrolidinophosphonium hexafluorophosphonate/iPr_2EtN coupling step, which resulted in a loading yield of 45%. All remaining amino acids were coupled by stepwise solid-phase synthesis by using 2-(6-chloro-1*H*-benzotriazole-1-yl)-1,3,3-tetramethylammonium hexafluorophosphate/iPr_2EtN activation protocols. Alkylation of the linker's sulfonamide functionality was achieved with iodoacetonitrile according to the procedure of Backes et al.[18] The peptides were liberated from the resin by adding a five-fold excess of TFA or the tosylate salt of H-Asp-SCm and H-Asp(O*t*Bu)-OGp(Boc,Boc) to provide the fully protected peptide carboxymethyl thioester or 4-guanidinophenyl ester. Neutralisation of the trifluoroacetate or tosylate was achieved by adding appropriate equivalents of *N*-methylmorpholine. Deprotection of the side-chain and guanidino functionalities by TFA treatment, and purification of the crude products by preparative HPLC resulted in the respective N^{α}-Z-protected derivatives. The identity and purity of the synthesis products were checked by analytical HPLC, NMR spectroscopy, elementary analysis, and mass spectrometry. In all cases, satisfactory analytical data were found (Supporting Information).

Solvent-jump experiments: Peptidyl prolyl *cis*/*trans* isomerisation was monitored in solvent-jump experiments by applying the established protocols that were developed for the LiCl/trifluoroethanol system.[11] Accordingly, the peptide anilides (75–88 mg, 1.6 mM) were dissolved in the ionic liquid system at room temperature. After 30 min incubation time, an aliquot (60 μL) was withdrawn and added to a tempered (10 °C) *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES) 0.035 M, pH 7.8 that contained chymotrypsin (2.6×10^{-5} M) (1840 μL). Subsequently the isomer-specific proteolysis was recorded at 390 nm, from which the *cis*/*trans* ratios and finally the reported $\Delta\Delta G$ values were calculated.[11]

Enzymatic syntheses: The enzymatic reactions were performed in a volume of 0.2 mL that contained 3-[*N*-morpholino]propanesulfonic acid (MOPS) 0.2 M, pH 8.0, 5 or 30% (*v*/*v*) DMF (aqueous medium) and 50, 60, and 70% (*v*/*v*) ionic liquid/MOPS mixtures. The peptides LIVNAVLQPVAAGAY and KVVFSCPVLEPTGPLHTQFGY-HIIKV LYRN, amino acid and peptide esters were dissolved in DMF (control reactions in aqueous medium) or ionic liquid whereas the pentapeptides were suspended in buffer. Readjusting to pH 8.0 was achieved by adding NaOH. After thermal equilibration of the assay mixtures at 25 °C (chymotrypsin and trypsin) or 37 °C (V8 protease), the reactions were initiated by the addition of enzyme stocks. After defined time intervals, the reactions were quenched by the addition of 1% TFA solution, and were subsequently analysed as described below. To check for spontaneous reactions, parallel reactions without enzyme were studied in all cases. On the basis of these controls, nonenzymatic reactions could be ruled out, and the extent of spontaneous hydrolysis of the acyl donor esters was found to be less than 5%. All reported data are the average of at least three independent reactions, and the errors were generally less than 5% (±2.5%).

Analyses: The reactions were analysed under optimised conditions by reversed-phase HPLC (Grom Capcell, 5 μm, 25×0.4 cm; Shiseido, Tokyo, Japan). Detection was carried out at 254 nm or 280 nm (17+30 fragment condensation). Mass spectra were recorded for isolated and lyophilised probes by using MALDI-ToF (MALDI 5V5.1.2, Kratos Kompakt, Manchester, UK) or ESI (Apex II/7 Tesla, Bruker Daltonics) ionisation. NMR spectroscopy (GEMINI 300, Varian) was used to verify the identity of the amino acid esters. CD spectra were recorded on a JASCO J-710 photometer.

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[1] a) P. E. Dawson, S. B. H. Kent, *Annu. Rev. Biochem.* **2000**, *69*, 923–960; b) J. P. Tam, Q. Yu, Z. Miao, *Peptide Sci.* **1999**, *51*, 311–332.
[2] P. E. Dawson, T. W. Muir, I. Clark-Lewis, S. B. Kent, *Science* **1994**, *266*, 776–779.
[3] B. L. Nilsson, L. L. Kiessling, R. T. Raines, *Org. Lett.* **2000**, *2*, 1939–1941.
[4] F. Bordusa, *Chem. Rev.* **2002**, *102*, 4817–4867.
[5] a) J. H. Davis, Jr., *Chem. Lett.* **2004**, *33*, 1072–1077; b) M. Koel, *Crit. Rev. Anal. Chem.* **2005**, *35*, 177–192; c) S. Lee, *Chem. Commun. (Cambridge)* **2006**, 1049–1063; d) J. M. Slattery, C. Daguenet, P. J. Dyson, T. J. S. Schubert, I. Krossing, *Angew. Chem.* **2007**, *119*, 5480–5484; *Angew. Chem. Int. Ed.* **2007**, *46*, 5384–5388; e) R. Ludwig, U. Kragl, *Angew. Chem.* **2007**, *119*, 6702–6704.
[6] a) T. Welton, *Chem. Rev.* **1999**, *99*, 2071–2083; b) R. Sheldon, *Chem. Commun. (Cambridge)* **2001**, 2399–2407; c) *Ionic Liquids in Synthesis* (Eds.: P. Wasserscheid, T. Welton), Wiley-VCH, Weinheim, **2002**; d) F. van Rantwijk, R. M. Lau, R. A. Sheldon, *Trends Biotechnol.* **2003**, *21*, 131–138; e) F. van Rantwijk, R. A. Sheldon, *Chem. Rev.* **2007**, *107*, 2757–2785.
[7] a) M. Erbeldinger, A. J. Mesiano, A. J. Russell, *Biotechnol. Prog.* **2000**, *16*, 1129–1131; b) G.-W. Xing, F.-Y. Li, C. Ming, L.-N. Ran, *Tetrahedron Lett.* **2007**, *48*, 4271–4274.
[8] a) M. Thormann, S. Thust, H.-J. Hofmann, F. Bordusa, *Biochemistry* **1999**, *38*, 6056–6062; b) R. Günther, S. Thust, H.-J. Hofmann, F. Bordusa, *Eur. J.*

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Biochem. **2000**, *267*, 3496–3501; c) N. Wehofsky, F. Bordusa, *FEBS Lett.* **1999**, *443*, 220–224.

[9] F. Bordusa, R. Rudolph, N. Wehofsky, *Methods for the Synthesis and Selective Biocatalytic Modification of Peptides, Peptide Mimetics, and Proteins* **2002**, Pat. No. DE 10240098.9, PCT/EP03/09694, U.S. 10/526,163.

[10] F. Bordusa, *Curr. Protein Pept. Sci.* **2002**, *3*, 159–180.

[11] a) J. L. Kofron, P. Kuzmic, V. Kishore, E. Colon-Bonilla, D. H. Rich, *Biochemistry* **1991**, *30*, 6127–6134; b) C. Garcia-Echeverria, J. L. Kofron, P. Kuzmic, V. Kishore, D. H. Rich, *J. Am. Chem. Soc.* **1992**, *114*, 2758–2759; c) C. Garcia-Echeverria, J. L. Kofron, P. Kuzmic, V. Kishore, D. H. Rich, *Biochem. Biophys. Res. Commun.* **1993**, *191*, 70–75; d) B. Janowski, S. Wöllner, M. Schutkowski, G. Fischer, *Anal. Biochem.* **1997**, *252*, 299–307.

[12] A. M. Klibanov, *Nature* **2001**, *409*, 241–246.

[13] L. Crowhurst, N. L. Lancaster, J. M. P. Arlandis, T. Welton, *J. Am. Chem. Soc.* **2004**, *126*, 11549–11555.

[14] G. Bell, A. E. M. Janssen, P. J. Halling, *Enzyme Microb. Technol.* **1997**, *20*, 471–477.

[15] a) G. Fischer, T. Aumüller, *Rev. Physiol. Biochem. Pharmacol.* **2003**, *148*, 105–150; b) C. Schiene-Fischer, C. Yu, *FEBS Lett.* **2001**, *495*, 1–6; c) C. Scholz, J. Rahfeld, G. Fischer, F. X. Schmid, *J. Mol. Biol.* **1997**, *273*, 752–762.

[16] A. Kuhlewein, G. Voll, B. Hernandez Alvarez, H. Kessler, G. Fischer, J. U. Rahfeld, G. Gemmecker, *Protein Sci.* **2004**, *13*, 2378–2387.

[17] S. S. Wang, *J. Am. Chem. Soc.* **1973**, *95*, 1328–1333.

[18] a) G. W. Kenner, J. R. McDermott, R. C. Sheppard, *J. Chem Soc. D* **1971**, 636–637; b) B. J. Backes, A. A. Virgilio, J. A. Ellman, *J. Am. Chem. Soc.* **1996**, *118*, 3055–3065; c) B. J. Backes, J. A. Ellman, *J. Org. Chem.* **1999**, *64*, 2322–2330.

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