Supplementary Material (ESI) for Chemical Communications

Supporting information for:

Soluble, folded and active subtilisin in a protic ionic liquid

Francesco Falcioni, Hazel R. Housden, Zhenlian Ling, Seishi Shimizu, Adam J. Walker and Neil C. Bruce

Methods / experimental details

Reagents. Traditional ILs were purchased from Merck, while the the (hydroxylalkylammonium) protic ionic liquids (PILs) were kindly donated by Bionqs Ltd (UK). These ionic liquids were made-to-order at Bioniqs standard analytical grade plus custom specifications of minimal water (using freeze-drying and molecular sieves) and reduced absorbance between 200 and 400 nm. Results with DEA Cl were successfully repeated with different batches of ionic liquid and by different personnel. The structures of the cations and anions that make up PILs used in this study are shown in figures S1 and S2, respectively. N-acetyl-L-phenylalanine (AP), N-acetyl-L-phenylalanine ethyl ester (APEE), N-acetyl-L-phenylalanine methyl ester (APME), Subtilisin A and α-chymotrypsin (type II, from bovine pancreas) were purchased from Sigma Aldrich Company, UK. All the molecular solvents used were of HPLC grade and were stored overnight with molecular sieves when required to be anhydrous.

Enzyme preparation. Enzymes were prepared in a similar manner to procedures described in the literature (J. A. Laszlo, D. L. Compton, *Biotechnol. Bioeng.* **2001**, 75, 181-186.), to provide the enzyme powder with the required "pH memory". The chosen mass of enzyme powder (typically 100 mg) was combined with an equal mass of K_2HPO_4 and then water was added to a final protein concentration of approx 10 g/l. The pH of this solution was decreased to 7.0 (chymotrypsin) or 7.5 (subtilisin) by the addition of concentrated KH_2PO_4 solution. To minimize autolysis, the solutions were kept on ice during the preparation and then snap-frozen in liquid nitrogen and freeze-dried as soon as possible, using a Thermo Heto LL3000 freeze dryer with a BOC Edwards RV8 vacuum pump. Enzymes prepared in this way exhibited improved dissolution in the ILs, which was generally a slow process, the precise time depending on the particular enzyme / solvent combination (typically a few hours for subtilisin in DEA Cl), with sonication or gentle heating for short periods to expedite this process. When the ionic liquid and enzyme powder were first combined a visible suspension was observed. Following the heating / sonication described above the enzyme was observed to dissolve, ie the system was no longer turbid. It is therefore possible that the 'dissolved' enzyme is not actually a molecular solution but a colloidal dispersion that appears to be a solution.

Effect of enzyme pre-treatment on activity and structure. Several different formulations of the subtilisin were prepared, and the effect of these formulations on enzyme activity and protein structure when dissolved in PILs were observed. It was found that powdered subtilisin as supplied by the manufacturer was not active when dissolved directly into DEA Cl, and had 'flat' CD spectra indicating a lack of native protein structure (data not shown). If the commercially available subtilisin powder was first dissolved in water (with or without the addition of buffer salts) and then freeze-dried, the resulting enzyme preparation was active when dissolved in DEA Cl and had native-like near and far CD spectra. Similarly, if the commercial subtilisin powder was combined with a small amount of water (1 % of final PIL volume) prior

to addition of and dissolution in the DEA Cl, the enzyme was also active and exhibited native-like CD spectra.

Enzyme assays. Reactions were conducted in vials chemically compatible with the used solvent (typically 5 ml glass vials with PTFE-lined lids) and equipped with magnetic stir bars. Vials were loaded with a known mass of enzyme preparation (typically 2 mg of enzyme) and 4 ml of reaction solvent (= 0.5 g/l of enzyme). The enzyme powders were dissolved in the ILs or in organic solvent or 50 mM K_2HPO_4/KH_2PO_4 buffer at pH 7.5 or 7.0, for subtilisin or chymotrypsin, respectively. Afterwards, the substrates APEE (typically 5 mM) and anhydrous 1-propanol (500 mM) were added, to start the transformation. Reactions were conducted in a constant-temperature (40 °C) water bath, with magnetic stirring in each vial.

At time points between 15 min and 24 hours, 200 µL aliquots were removed, diluted with 800 µl DMSO and centrifuged at 9500×g for 60 s to remove suspended particles. APEE and the products of the reaction, APPE, APME and AP, were separated and quantified by HPLC. HPLC analysis was performed with a Waters 510 Pump, equipped with a Waters 2487 dual wavelength absorbance detector and a Waters 717 autosampler. Data were treated with the "Empower Pro" software (Waters). Separation was performed on a Phenomenex Prodigy C8, 250 x 4.60 mm column, with 5 micron particles, using eluent 60% aqueous acetic acid (1% v/v) 40% acetonitrile, flow rate 1 ml/minute (isocratic), injection volume 10 µl, with spectrophotometric detection at 258 nm. Retention times were approximately: 4.1 minutes (AP), 5.7 minutes (APME), 7.2 minutes (APEE), 11.2 minutes (APPE) and 18.1 minutes (APBE). Authentic standards were only commercially available for AP, APEE and APME. The chromophore in these molecules is the phenyl ring, which is virtually untouched in the different compounds, and the calibration curves designed for these three compounds revealed very similar extinction coefficients, to the point that the values could be arbitrarily extrapolated also to APPE, for which no standards were available. Retention times for these compounds were determined by observing product formation for the reactions in dry hexane. These retention times and the exact nature of the products were subsequently confirmed by LC-MS-MS.

LC-MS-MS analysis. Reactions were set up with 50 mM APEE, 500 mM propanol and 2 g/l enzyme and incubated at 40 °C for 60 min. Samples were extracted with a mixture of acetonitrile and water (100 µl sample, 400 µl acetonitrile, 30 µl water). The resulting (mainly organic) upper phase was analyzed by LC-MS-MS.

LC-MS-MS was performed with an Applied Biosystems QSTAR Pulsar i instrument, electrospray ionization, equipped with a Zorbax Eclipse XDB C8 column (5 µm, 150 × 4.6 mm; Agilent); using eluent 70 % aqueous acetic acid (1% v/v) 30% acetonitrile, pumped isocratically at 1 ml/min.

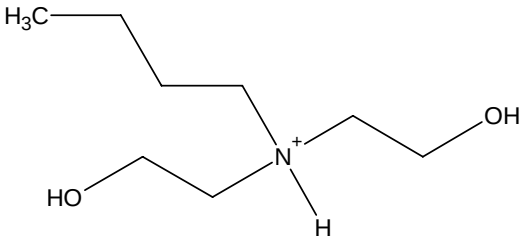
To identify the unknown compound eluting at 2.3 mins, the profile of the main ion (MS) and it's fragmentation (MS-MS) (Figure S3a and b, respectively) were examined. ChemSketch software was used to draw the structures of predicted and possible products of the reaction and confirm the monoisotopic masses (Figure S4). The weaker bonds were then identified and from these the theoretical fragments were designed and their monoisotopic masses calculated. Comparison of these masses with the fragmentation profiles from the secondary MS data (Figure S3b) led to identification of the unknown products beyond reasonable doubt.

Water content and water activity measurements. Water contents of the solvents were measured by volumetric Karl Fischer moisture titration, using a KEM Karl Fischer Moisture Titrator MKS-500 (Integrated Scientific Ltd.), with Hydranal Composite 5 as titrant and Hydranal Solvent as sample solvent (Riedel-de Haën, catalogue numbers 34805 and 34800). Between 70 and 200 µl of material was sampled with a positive-displacement pipette (Gilson Microman M100 or M250) which was weighed on a precision balance (± $1x10^{-4}$ g) before and after dispensing the sample into the analysis chamber. The water contents were reported as a (w/w) percentage.

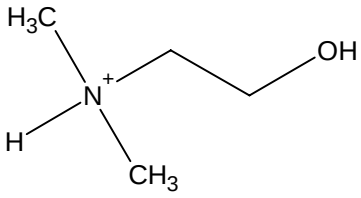
Water activity of the samples was measured with a Rotronic HygroPalm 1 instrument. Aliquots of 1 to 4 ml were placed in a small, covered Petri dish (provided with the instrument) forming a sealed chamber. Samples were allowed to equilibrate (10 to 30 min), and then the values were recorded.

Circular dichroism. Circular dichroism was performed on a Jasco J810 CD spectrophotometer. Near UV spectra were collected with a 5 mm path length cuvette containing 0.7 to 2 g/l of enzyme. Far UV spectra were collected with a 0.1 mm path length cuvette containing 0.7 to 2 g/l of enzyme. Spectra were collected using 2 nm bandwidth, 1 s response, 0.5 nm data pitch, 100 nm/min scan speed and 3 accumulations at 20°C. Blank spectra of solvent alone were recorded and subtracted from those of the corresponding samples. CD units are mean residue ellipticity ($[\theta]_{MRW}$)

FIGURE S1: protic alkylammonium cations



N,*N*-butyldiethanolammonium



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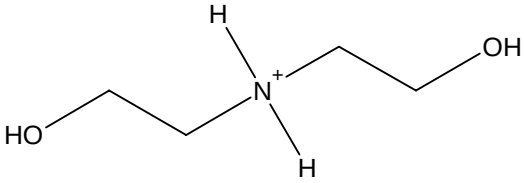
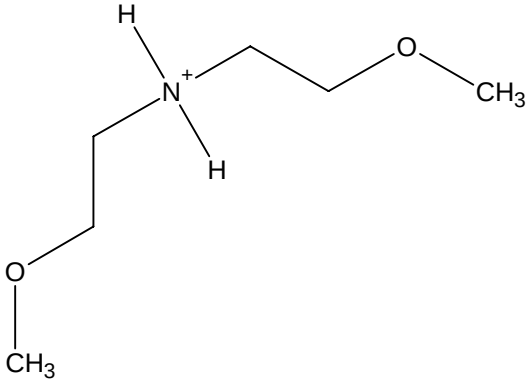
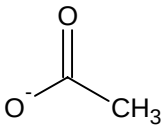


diethanolammonium



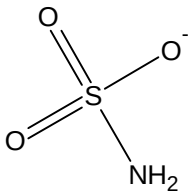
bis(2-methoxyethyl)ammonium

FIGURE S2: anions used in combination with hydroxylalkylammonium cations

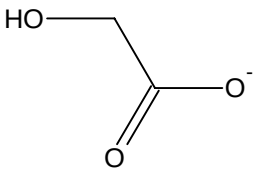


acetate

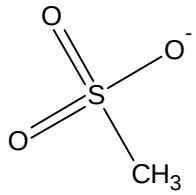
chloride



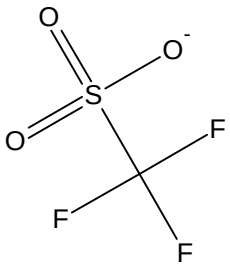
sulfamate



glycolate



methanesulfonate



trifluoromethanesulfonate

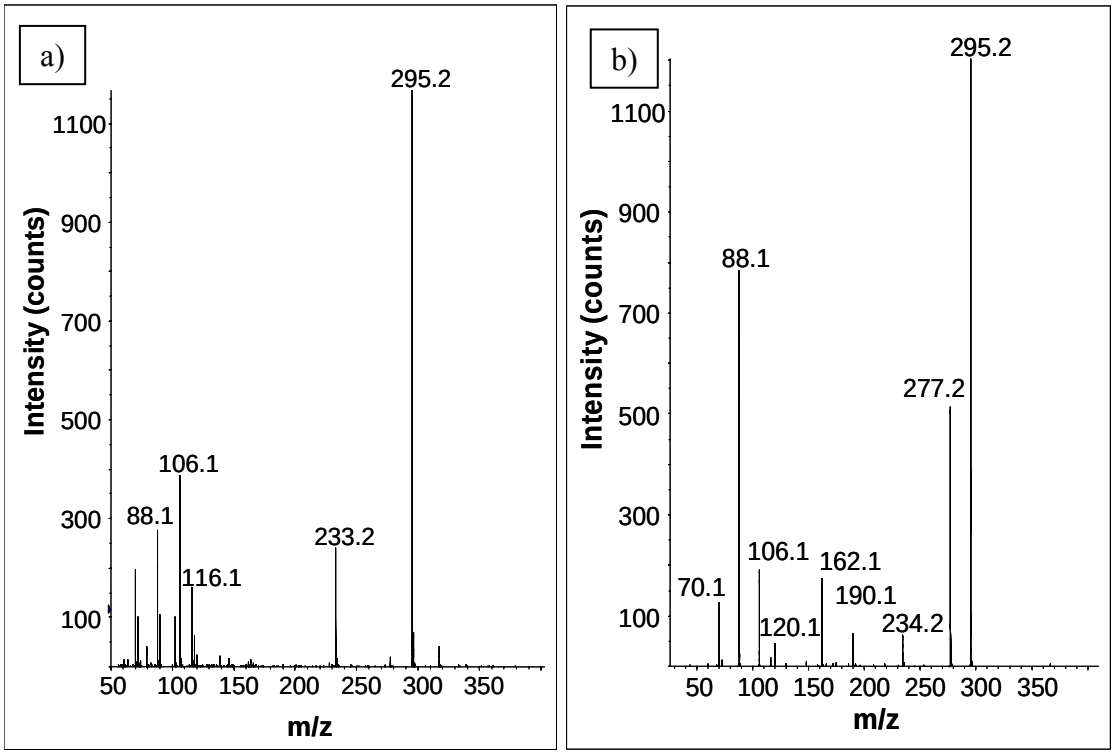
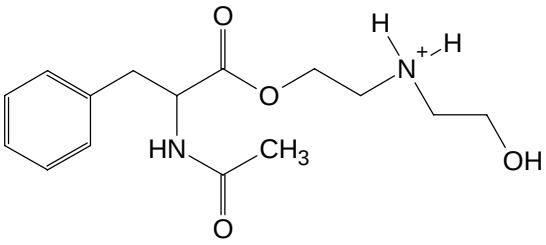
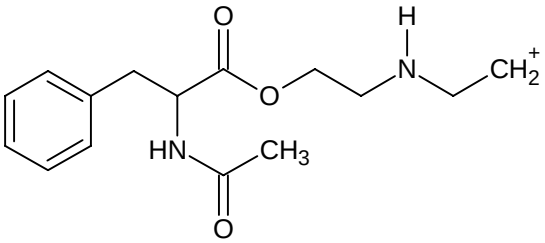


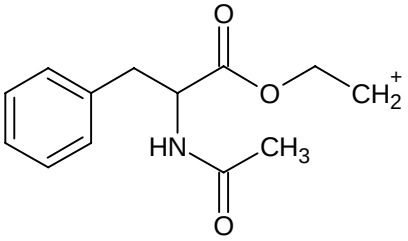
Figure S3: MS data for the peak at 2.3 min in figure 2 (of the Communication). a) Main ion profile and b) fragmentation profile (CE=20V) for the peak at 2.3 min in the HPLC analysis of the subtilisin-catalyzed reaction of APEE in pure DEA Cl with added propanol. Ions relevant to the identification are labeled with their m/z value.



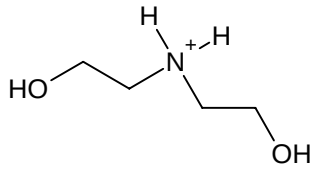
Monoisotopic Mass = 295.17 Da



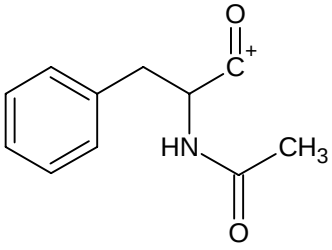
Monoisotopic Mass = 277.15 Da



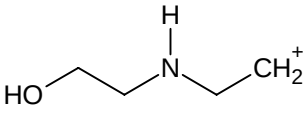
Monoisotopic Mass = 234.11 Da



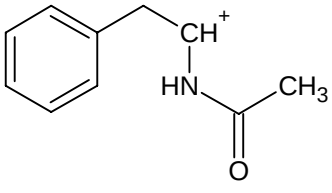
Monoisotopic Mass = 106.09 Da



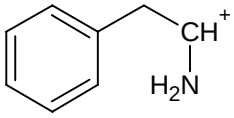
Monoisotopic Mass = 190.09 Da



Monoisotopic Mass = 88.08 Da



Monoisotopic Mass = 162.09 Da



Monoisotopic Mass = 120.08 Da

Figure S4: Proposed structure assignment and fragmentation pattern of the MS peak described in figure S3.
The peak elutes at 2.3 minutes in the HPLC analysis of the subtilisin-catalyzed reaction of APEE in pure DEA Cl and has a base peak of 295 m/z.