Supplementary Material (ESI) for Green Chemistry

Impact of ionic liquids on papain: an investigation of structure-function relationships

Wen-Yong Lou,[ab] Min-Hua Zong,*[a] Thomas J. Smith,[c] Hong Wu[a] and Ju-Fang Wang[a]
[a]*Laboratory of Applied Biocatalysis, South China University of Technology, Guangzhou 510640, China. Fax: +86-20-2223-6669; Tel: +86-20-8711-1452; E-mail: btmhzong@scut.edu.cn (Prof. M. H. Zong); wylou@scut.edu.cn (Dr. W.Y. Lou)*
[b]*The State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai 200237, China.*
[c]*Biomedical Research Centre, Sheffield Hallam University, Owen Building, Howard Street, Sheffield, S1 1WB, UK. E-mail: t.j.smith@shu.ac.uk (T. J. Smith)*

Electronic Supplementary Information (ESI)

Experimental section

Enzymatic reaction

D,L-*p*-hydroxyphenylglycine methyl ester (D,L-HPGME, 0.6 mmol) was added to screw-capped vials containing 4 ml of the co-solvent system of phosphate buffer (50 mM, pH 7.0) and IL ($C_2MIm•BF_4$, $C_3MIm•BF_4$, $C_4MIm•BF_4$, $C_5MIm•BF_4$, $C_6MIm•BF_4$, $C_4MIm•HSO_4$, $C_4MIm•Cl$, $C_4MIm•NO_3$ or $C_4MIm•CH_3COO$, being present at 15% by volume) or the pure phosphate buffer. The reaction was started by addition of 600 U papain (12 U/mg) and run at 45 ^oC in a water-bath shaker with a shaking rate of 200 rpm. Aliquots (50 μl) were withdrawn at specified time intervals from the reaction mixture and diluted with 950 μl of 0.2% (v/v) formic acid for HPLC analysis. To assay kinetic parameters, the reactions were carried out with D,L-HPGME concentrations between 10 and 75 mM. The apparent kinetic parameters (K_m, V_{max}) were estimated by fitting the data to a standard Michaelis-Menten equation using the Eadie-Hofstee plot ($v=V_{max} - K_m \bullet v/c_s$, v: initial reaction rate; c_s: substrate concentration).

HPLC analysis

The reaction mixture was analyzed by HPLC (Waters, USA) on a 4 ×150 mm 5 μ Crownpak CR (+) chiral column from Daicel Chemical Industries Co., Ltd (Tokyo, Japan) using a Waters 600 pump and a Waters 996 Photodiode Array Detector at 228 nm. The mobile phase was an aqueous solution of $HClO_4$ (11mM, pH 2.0) at 0.8 ml/min. The retention times for D-*p*-hydroxyphenylglycine, D-*p*-hydroxyphenylglycine methyl ester, L-*p*-hydroxyphenylglycine and L-*p*-hydroxyphenylglycine methyl ester were 2.78, 5.23, 9.32 and 25.54 min, respectively. The initial reaction rate (or activity), enantiomeric excess of remaining substrate (ee_s), substrate

conversion (C) and enantioselectivity (E-value=ln [(1 – C)(1 – ee_s)]/ ln [(1 – C)(1 + ee_s)]) were calculated from the HPLC data. The average error for this determination was less than 0.7%. All reported data are averages of experiments performed at least in duplicate.

ATR-FTIR spectroscopic analysis

ATR-FTIR spectra were recorded from 4000 to 600 cm^{-1} using a Bruker Tensor 27 instrument equipped with a MCT detector as the average of 32 scans at 2 cm^{-1} resolution. The system was managed by Bruker OPUS software. Approximately 20 μl of solution of papain (5 mg/ml) in the co-solvent system of phosphate buffer (50 mM, pH 7.0) and IL ($C_2MIm•BF_4$, $C_3MIm•BF_4$, $C_4MIm•BF_4$, $C_5MIm•BF_4$, $C_6MIm•BF_4$, $C_4MIm•HSO_4$, $C_4MIm•Cl$, $C_4MIm•NO_3$ or $C_4MIm•CH_3COO$, being present at 15% by volume) or in pure phosphate buffer （50 mM, pH 7.0） was pipetted onto a Specac Golden Gate diamond ATR window for ATR-FTIR measurements. The reference spectra (or the background spectra) were recorded under identical conditions with only the solvent systems. The protein spectra were corrected by subtraction of the reference spectra, and were smoothed with a 9-point Savitsky-Golay method to remove white noise. The second derivative spectra in the amide I region of papain were achieved with the Savitsky-Golay derivative function for a nine data point window with spectra analysis software (Bruker OPUS).

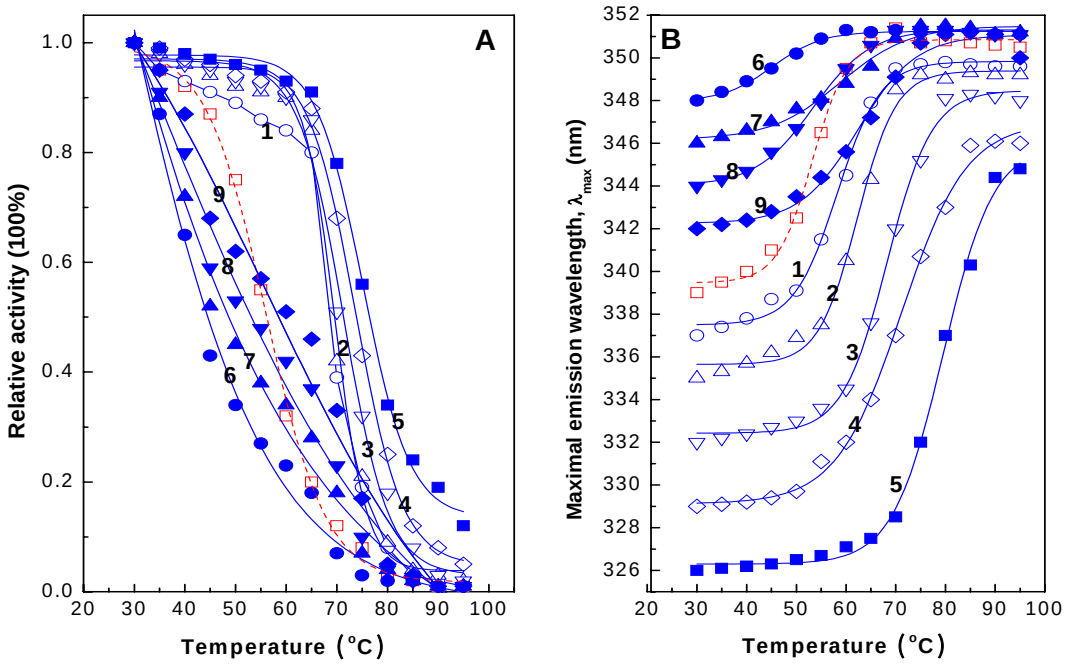
Fluorescence spectroscopic analysis

Intrinsic fluorescence emission spectra of papain were monitored using a Hitachi spectrofluorometer (model F4500). Enzyme samples were pre-incubated in the cell for 5 min before being excited at 285 nm, and emission was recorded from 290 nm to 450 nm at a scan rate of 1200 nm/ min using a 5 nm bandwidth in both the excitation and emission paths. The final concentration of papain in the co-solvent system of phosphate buffer (50 mM, pH 7.0) and IL ($C_2MIm•BF_4$, $C_3MIm•BF_4$, $C_4MIm•BF_4$, $C_5MIm•BF_4$, $C_6MIm•BF_4$, $C_4MIm•HSO_4$, $C_4MIm•Cl$, $C_4MIm•NO_3$ or $C_4MIm•CH_3COO$, being present at 15% by volume) or in pure phosphate buffer was 0.1 mg/ml. The spectrofluorometer automatically provided corrected spectra by comparison with a 1 nM standard solution of rhodamine B in glycerol to correct for changes in lamp output and instrument geometry. The maximum emission wavelength of the sample was determined as the wavelength for which $dI/d\lambda=0$. In all cases, the fluorescence spectra of papain were obtained by subtracting the spectra of the solvents without the enzyme (or blank media).

Thermal stability of papain

Into different screw-capped vials containing 2 ml of the co-solvent systems of phosphate buffer (50 mM, pH 7.0) and ILs ($C_2MIm•BF_4$, $C_3MIm•BF_4$, $C_4MIm•BF_4$, $C_5MIm•BF_4$, $C_6MIm•BF_4$, $C_4MIm•HSO_4$, $C_4MIm•Cl$, $C_4MIm•NO_3$ or $C_4MIm•CH_3COO$, being present at 15% by volume) or pure phosphate buffer, papain (10 mg) was added and incubated for 60 min at a temperature varied from 30 °C to 95 °C. The substrate (0.3 mmol D,L-HPGME) was then added to each vial, and the enzymatic hydrolysis reaction was followed as described above for the measurement of the residual activity.

Fig. S1 Temperature-dependent activity decay (**A**) and maximal emission wavelength (**B**) of papain in the systems involving ILs **1-9**‡ (blue curve) and the aqueous buffer control (red curve). Samples were analysed after 60 min incubation at each temperature; residual enzyme activity was assayed at 30 ºC as described above.



Effect of IL concentration on stability of papain

Aliquots of papain (10 mg) were incubated for 60 min at 45 ^{o}C in screw-capped vials containing 2 ml of the various co-solvent systems, which comprised ILs ($C_2MIm•BF_4$, $C_3MIm•BF_4$, $C_4MIm•BF_4$, $C_5MIm•BF_4$, $C_6MIm•BF_4$, $C_4MIm•HSO_4$, $C_4MIm•Cl$, $C_4MIm•NO_3$ or $C_4MIm•CH_3COO$) at a range of concentrations (0~50%, v/v) in phosphate buffer (50 mM, pH 7.0). Substrate (0.3 mmol D,L-HPGME) was then added to each vial, and the enzymatic hydrolysis reaction was followed as described above.

Fig. S2 Effect of IL (**1-9**‡) concentration on the stability of papain. Assays were performed after 60 min incubation at 45 ^{o}C.