

# Liquid–liquid extraction of lysozyme using a dye-modified ionic liquid

Yu-Ping Tzeng, Ching-Wei Shen, Tiing Yu\*

*Department of Applied Chemistry, National Chiao Tung University, Hsinchu 30050, Taiwan*

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## Abstract

An affinity-dye, Cibacron Blue 3GA (CB), derivatized organic salt [BMIM]<sub>3</sub>[CB] was synthesized for lysozyme extraction. This compound was formed by mixing an ionic liquid (IL) [BMIM][Cl] and the silver salt of CB. Liquid–liquid extractions of lysozyme from the aqueous and [BMIM]<sub>3</sub>[CB] in [BMIM][PF<sub>6</sub>] solutions were examined in this study. The transfer of lysozyme from the aqueous phase to the IL phase decreased while the pH of the aqueous phase increased. An extraction higher than 90% was observed at pH 4. Under a high ionic strength, the lysozyme would transform back from the IL phase into the aqueous phase. Lysozyme molecules were almost quantitatively recovered from the IL phase to the aqueous solutions of 1 M KCl under pH 9–11. It appeared that the extraction was specific for lysozyme in contrast to cytochrome *c*, ovalbumin, and bovine serum albumin. The extraction efficiency of the IL phase remained essentially the same after eight cycles of extraction. © 2008 Elsevier B.V. All rights reserved.

**Keywords:** Ionic liquid; Affinity extraction; Protein purification; Cibacron Blue 3GA; Lysozyme

## 1. Introduction

Ionic liquids (ILs) are molten organic salts which exhibit many attractive properties such as negligible volatility, non-flammability, thermal stability, and selective solubility. They are used in liquid–liquid extractions and are considered to be environmentally benign solvents because of their very low vapor pressures. The designable solubilities for many organic molecules can sometimes be achieved by altering the cationic or anionic moieties of the ILs. For example, the hydrophobicity of IL can be enhanced by increasing the alkyl length of cationic ions [1]. In liquid-phase microextraction for polycyclic aromatic hydrocarbons, 1-octyl-3-methylimidazolium hexafluorophosphate ([C<sub>8</sub>MIM][PF<sub>6</sub>]) provided a higher enrichment factor than those of 1-butyl-3-methylimidazolium hexafluorophosphate ([C<sub>4</sub>MIM][PF<sub>6</sub>], also known as [BMIM][PF<sub>6</sub>]) and 1-hexyl-3-methylimidazolium hexafluorophosphate ([C<sub>6</sub>MIM][PF<sub>6</sub>]) [2]. In addition, [BMIM][PF<sub>6</sub>] has been applied to the direct extraction of DNA via the interaction between cation BMIM<sup>+</sup> and the phosphate groups of DNA [3].

The liquid–liquid extractions of ionic dyes from water have been performed using an imidazolium-based ionic liquid [4,5]. The reversible liquid–liquid partitions of anionic dyes were strongly affected by the pH of the aqueous phase. In addition, ILs modified with crown ethers have been applied in the extraction of metal ions [6,7] and biomaterials such as amino acids [8]. Goto and co-workers reported that the Lys-rich protein cytochrome *c* [9,10] could be transferred to the hydroxyl group-containing ILs with crown ethers via supramolecular complexation.

Dye–ligand affinity is a specific interaction between proteins and reactive dyes. The biospecific adsorption results from the polarity and geometry of the reactive dye. Cibacron Blue 3GA (CB) is a triazine-containing dye and has been found to be very effective in the purification of NAD-utilizing enzymes [11]. It can be immobilized on the surface of an agarose matrix [12] or porous polystyrene particles [13] to produce affinity-chromatography columns for enzyme purification. After sample injection, enzyme molecules are bound effectively to the column. Proteins without the specific affinity adsorption would be eluted from the column. The desired enzyme is then eluted out of the column and collected with a mobile phase of high salt concentration. In addition, the CB dye can be immobilized on magnetic particles [14,15] and hollow-fiber membranes [16] for lysozyme purification. The dye–ligand affinity based on aqueous two-phase extraction provided a low-cost procedure

\* Corresponding author. Tel.: +886 3 573 1673; fax: +886 3 572 3764.  
E-mail address: [tyu@faculty.nctu.edu.tw](mailto:tyu@faculty.nctu.edu.tw) (T. Yu).

and large-scale preparation for the enzymes [17]. The reverse micelle system works as another medium for dye–ligand affinity in liquid–liquid extraction of lysozyme [18,19]. Surfactants modified with CB were utilized in the purification of lysozyme with high efficiency.

In this study, we synthesized a CB-derivatized organic salt. With this organic salt as an extractant in [BMIM][PF<sub>6</sub>], lysozyme can be efficiently extracted back and forth between water and the IL phases.

## 2. Experimental

### 2.1. Materials

Cytochrome *c* from horse heart, lysozyme and ovalbumin from chicken egg white, bovine serum albumin (BSA), and Cibacron Blue F-3GA were all purchased from Sigma (St., Louis, Mo, USA), while 1-chlorobutane (GR grade) and 1-methylimidazole (GR grade) were purchased from Acros (Fairlawn, NJ, USA). Potassium hexafluorophosphate, potassium chloride, sodium acetate, sodium dihydrogenphosphate anhydrous, potassium dihydrogenphosphate, and sodium hydrogen carbonate from Showa (Tokyo, Japan) were all reagent grades. Glycine was purchased from Riedel-deHaen (Seelze, Germany), while trifluoroacetic acid (TFA) was from Alfa Aesar (Ward Hill, MA, USA). Ethyl acetate, acetonitrile, methanol, and acetone were all of HPLC grade and were obtained from Tedia (Fairfield, OH, USA). De-ionized water was purified from Millipore Milli-Q plus (Millipore, Bedford, MA, USA).

### 2.2. Synthesis of [BMIM][PF<sub>6</sub>]

The IL [BMIM][Cl] (1-butyl-3-methylimidazolium chloride) was prepared by mixing 0.6 mole of 1-chlorobutane and 0.6 mole of 1-methylimidazole in a flask, followed by stirring and heating of the solution at 80 °C for 48 h with reflux. A viscous yellow liquid was obtained and cooled at room temperature and was washed 3 times with 150 ml of ethyl acetate. After washing, the remaining ethyl acetate was removed by heating under vacuum. Preparation of [BMIM][PF<sub>6</sub>] [20] was then carried out by mixing equal molar amounts of [BMIM][Cl] and KPF<sub>6</sub> (0.6 mole) in 300 ml of water at room temperature. Two immiscible phases were formed after stirring for 6 h. The upper aqueous phase was removed, and the lower IL phase was washed several times with 300 ml of water until the pH of the aqueous phase remained nearly unchanged. The lower IL phase was heated under vacuum to remove the remaining water.

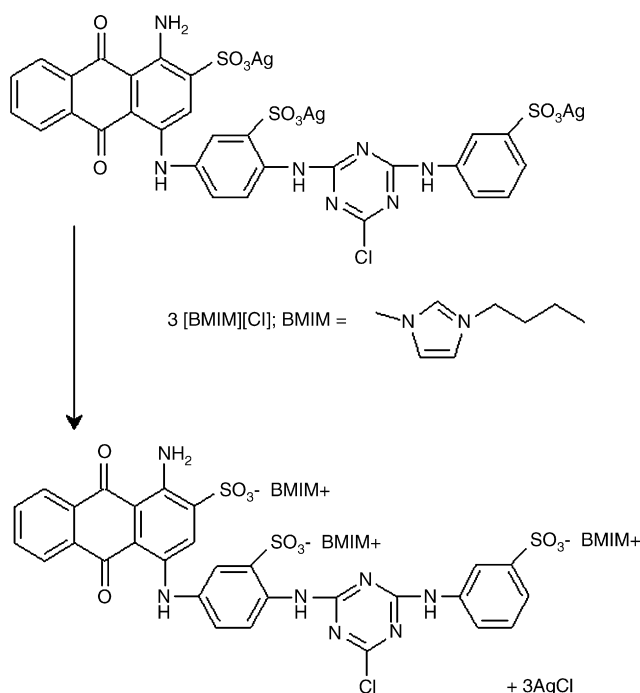
### 2.3. Synthesis of [BMIM]<sub>3</sub>[CB]

In 50 ml de-ionized water, 1.68 g ( $2 \times 10^{-3}$  mole) of CB and 3.39 g ( $2.0 \times 10^{-2}$  mole) of AgNO<sub>3</sub> were dissolved and stirred for 24 h. After centrifuging ( $3400 \times g$ , 5 min), the upper aqueous phase was removed, and the blue precipitate of silver salt was washed for 3 times with 10 ml of ice water. The precipitate (yield: 2.01 g, 92%) was dried under vacuum and then was dissolved in 10 ml of acetonitrile. The solution was mixed

with three equivalents of [BMIM][Cl] under 40 °C. After stirring for 72 h, [BMIM]<sub>3</sub>[CB] was formed with precipitation of AgCl. The reaction is shown in Scheme 1. After AgCl precipitate was removed by centrifuging ( $3400 \times g$ , 5 min). Then the acetonitrile solution was heated under vacuum to obtain the crude organic salt. The crude product was dissolved in acetone to separate from the unreacted precursor Ag<sub>3</sub>CB. The organic salt (yield: 1.18 g, 54%) recovered from the acetone solution under vacuum was identified by <sup>1</sup>H NMR and ESI-MS. The <sup>1</sup>H NMR spectra of the ILs were measured using Uniytinova-500 (Varian, USA).

<sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) of [BMIM][Cl]:  $\delta$ : 9.63 (NCHN), 7.92 (CH<sub>3</sub>NCHCHN), 7.83 (CH<sub>3</sub>NCHCHN), 4.20 (NCH<sub>2</sub>(CH<sub>2</sub>)<sub>2</sub>CH<sub>3</sub>), 3.87 (NCH<sub>3</sub>), 1.74 (NCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 1.21 (N(CH<sub>2</sub>)<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 0.85 (N(CH<sub>2</sub>)<sub>3</sub>CH<sub>3</sub>). <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) of [BMIM]<sub>3</sub>[CB]:  $\delta$ : 9.14 (NCHN), 7.76 (CH<sub>3</sub>NCHCHN), 7.69 (CH<sub>3</sub>NCHCHN), 4.10 (NCH<sub>2</sub>(CH<sub>2</sub>)<sub>2</sub>CH<sub>3</sub>), 3.83 (NCH<sub>3</sub>), 1.74 (NCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 1.24 (N(CH<sub>2</sub>)<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 0.88 (N(CH<sub>2</sub>)<sub>3</sub>CH<sub>3</sub>).

The CB-containing IL [BMIM]<sub>3</sub>[CB] was a deep blue viscous liquid. Some small chemical shifts of [BMIM] were observed between the <sup>1</sup>H NMR spectra of [BMIM]<sub>3</sub>[CB] and [BMIM][Cl] due to the effect of different anions [21]. Accordingly, the BMIM moiety should remain intact during the synthesis. The mass spectrum of [BMIM]<sub>3</sub>[CB], obtained using an ESI-Quattro Micro Mass spectrometer (Waters, USA), showed a clear signal of BMIM<sup>+</sup> at *m/z* 139 under the positive mode. A significant signal of a trimolecular cluster [(BMIM)<sub>2</sub>CB]<sup>-</sup> at *m/z* 1048, a dimolecular cluster [(BMIM)(CB)]<sup>2-</sup> at *m/z* 454.5, and a doubly charged CB<sup>2-</sup> at *m/z* 385.5 were detected using the negative mode. The melting point, measured using a Du Pont TA 2000 (TA Instrument,



Scheme 1. Synthesis of [BMIM]<sub>3</sub>[CB].

New Castle, DE, USA) differential scanning calorimeter, was 8.4 °C.

#### 2.4. Determination of [BMIM]<sub>3</sub>[CB] concentration in the extraction phase

The anion species of ILs usually have significant effects on the solubility in aqueous solution [22]. The solubility of [BMIM][PF<sub>6</sub>] in water is 1.88 g/100 ml under room temperature [6]; this would cause some loss of the IL phase during the extraction. In addition, [BMIM]<sub>3</sub>[CB] was more soluble in water than [BMIM][PF<sub>6</sub>] due to the water-soluble CB moiety. During the protein extraction from the aqueous phase to the IL phase ([BMIM]<sub>3</sub>[CB] in [BMIM][PF<sub>6</sub>]), blue [BMIM]<sub>3</sub>[CB] also appeared in the aqueous phase. It was found that [BMIM]<sub>3</sub>[CB] could be better retained in the IL phase during the extraction by washing it several times with water before the extraction. This might occur partly due to water saturation in the IL phase that helped maintain the IL phase from being washed down by the aqueous sample solution. Therefore, all IL solutions were washed with water (equal volume) 5 times before being used for extractions. After washing, a 100 μl aliquot of the IL solution was pipetted and was added to 4 ml methanol. The absorbance at 623 nm of the resultant solution was determined using an Agilent 8453 UV–vis spectrometer (Waldronn, Germany). The calibration curve was done by the absorbance measurements of six different concentrations (0.25, 0.5, 1.25, 2.5, 5, and 10 mM) of [BMIM]<sub>3</sub>[CB] in [BMIM][PF<sub>6</sub>].

#### 2.5. Protein extractions

The protein sample solutions (500, 1000 mg/l) were prepared by dissolving a suitable amount of lysozyme in a buffer (50 mM of CH<sub>3</sub>COOH/CH<sub>3</sub>COONa, pH 4). Six solutions of [BMIM]<sub>3</sub>[CB] in [BMIM][PF<sub>6</sub>] were prepared, and the [BMIM]<sub>3</sub>[CB] concentrations were determined to be 0.28, 0.61, 1.55, 2.52, 3.8, and 5.82 mM, respectively. The stirring time was varied from 30 s to 60 min and the pH of aqueous phases was adjusted according the follow experiments.

Then an aliquot of 2 ml of each IL solution was pipetted and mixed with an equal volume of the lysozyme sample solution in a vial. After stirring for require time while the rotation speed of magnetic stir-bar was set at 400 rpm, the lysozyme concentration in the upper aqueous phase was measured using the HPLC.

#### 2.6. Analysis for proteins

The determination of protein concentrations was carried out using an HPLC system, equipped with a LabAlliance Series III pumping system (State College, PA, USA), an ODS column (150 mm × 4.6 mm, 10 μm) from Polymer Laboratories (Amherst, MA, USA), and a Bio-Rad Model 1801UV (Hercules, CA, USA) detector. The analysis was performed using a linear gradient at a flow rate of 1 ml/min from 0 to 100% mobile phase B in 20 min (mobile phase A: 20% acetonitrile and 0.1% TFA in water; mobile phase B: 80% acetonitrile and 0.1% TFA in water). Online signal was acquired using a SISC

version 3.1 Chromatography data station (Taipei, Taiwan). Protein standards were run to obtain a calibration curve, and the protein concentrations in the aqueous portion in the extraction experiment were quantitatively determined by peak area. In addition to the quantitative analysis, the secondary structures of the lysozyme solutions were examined in the far-UV range from 190 to 270 nm using a Jasco J-815 spectrometer (Tokyo, Japan) circular dichroism (CD) spectroscopy.

### 3. Results and discussion

#### 3.1. Optimization of extraction: [BMIM]<sub>3</sub>[CB] concentration and extraction time

The forward extraction efficiency as a function of [BMIM]<sub>3</sub>[CB] concentrations in the IL phase is shown in Fig. 1. The partition of lysozyme in the IL phase was influentially raised in the presence of [BMIM]<sub>3</sub>[CB] and the extraction increased as the concentration of [BMIM]<sub>3</sub>[CB] in the IL solution was elevated. It reached the state of equilibrium when the concentration of [BMIM]<sub>3</sub>[CB] was 3.8 mM. This concentration was larger by 100 times than those of the lysozyme concentrations (500 mg/l) in the sample solutions. Thus the concentration of [BMIM]<sub>3</sub>[CB] at 3.8 mM was used in the rest of the experiments.

Extractions were conducted under seven different stirring times, that is, 0.5, 1, 5, 10, 20, 30, and 60 min, for the lysozyme samples of 500 mg/l, and the result is shown in Fig. 2. The extraction reached 78% in 30 s of stirring time and above 95% in 10 min. The extraction attained the state of equilibrium in 30 min; hence, we conducted the rest of the experiments under the 30 min stirring time.

The aqueous portion after the forward and back extractions in an experiment was allowed to pass through a 5 ml HiTrap desalting column. The secondary structures of this solution and the standard lysozyme solution were measured using CD spectrometer. No significant change, as seen in Fig. 3, was observed

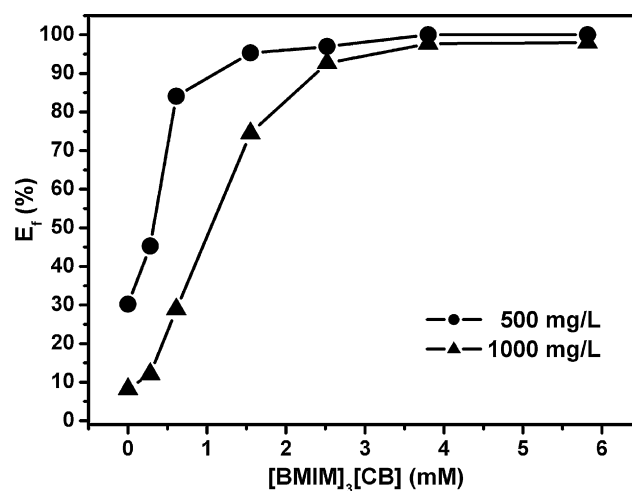


Fig. 1. Forward extractions under various [BMIM]<sub>3</sub>[CB] concentrations (0, 0.28, 0.61, 1.55, 2.52, 3.8, and 5.82 mM) in the [BMIM][PF<sub>6</sub>] phase. Two lysozyme concentrations (500, and 1000 mg/l in pH 4 buffer) of the aqueous phase were extracted in the experiment.

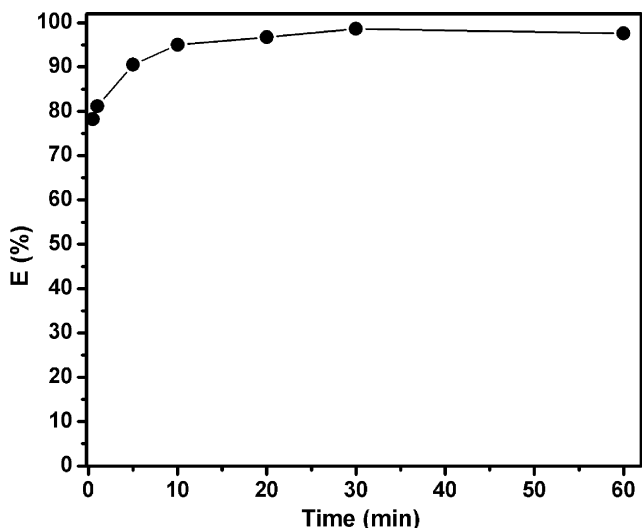


Fig. 2. Forward extraction of lysozyme to the IL phase as a function of stirring time. The stirring times were 0.5, 1, 5, 10, 20, 30, and 60 min, respectively, for lysozyme solutions of 500 mg/l in pH 4 buffer.

between these two spectra. Lysozyme molecules remained intact during the extraction procedures.

### 3.2. The partitioning isotherm for extraction capacity

Lysozyme solutions of different concentrations were extracted by the IL extraction solutions containing 3.8 mM [BMIM]<sub>3</sub>[CB] in order to examine the extraction capacity. The relationship of concentrations between extracted lysozyme ( $C_{IL}$ ) and lysozyme remaining in the aqueous phase ( $C_{aq}$ ) at equilibrium is shown in Fig. 4. Fitting these data points with Langmuir equation to obtain the partitioning isotherm:

$$C_{IL} = \frac{C_{IL,M}C_{aq}}{K_d + C_{aq}}$$

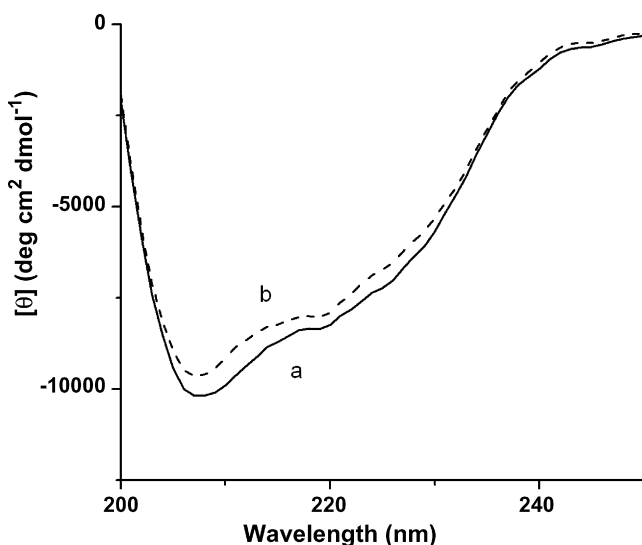


Fig. 3. Far-UV CD spectra of lysozyme in water (concentration  $\approx 0.1$  mg/l) (a), and recovered lysozyme (b). Spectra were measured at 25 °C.

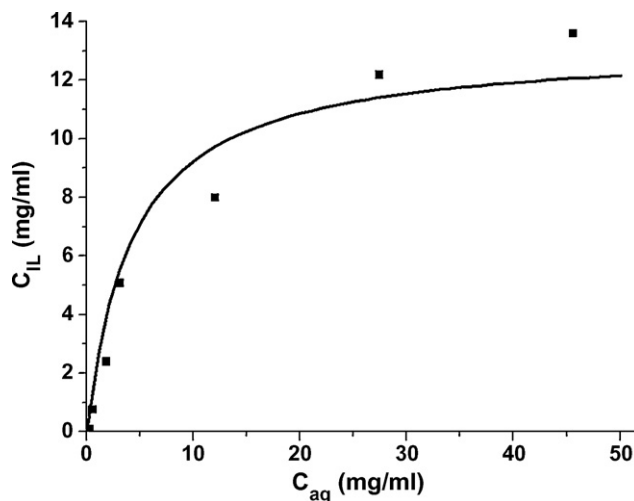


Fig. 4. The partitioning isotherm for extraction capacity. Concentration of the extracted lysozyme ( $C_{IL}$ ) to the IL phase is plotted as a function of lysozyme concentration remaining in the aqueous phase ( $C_{aq}$ ) at equilibrium.

the dissociation constant  $K_d$  obtained was  $\sim 4.29$  mg/ml and the extraction capacity  $C_{IL,M}$  was  $\sim 13.19$  mg/ml. Although the extraction data did not fit Langmuir equation very well, the large  $K_d$  value still implied a weak affinity between the protein molecule and the extraction solution. Accordingly, a very high concentration of [BMIM]<sub>3</sub>[CB] was required to achieve reasonably good extractions. Since the CB affinity sites in [BMIM]<sub>3</sub>[CB] were not bonded to the extraction phase, the extraction would depend on not only the CB-lysozyme affinity but also the solvation strength to solubilize the lysozyme-[BMIM]<sub>3</sub>[CB] complex into the IL phase. A liquid-liquid partitioning mechanism was suggested in the extraction instead of a typical Langmuir adsorption.

### 3.3. The pH effect on the forward and back extractions

In order to examine the effect of pH, lysozyme (500 mg/l) solutions were prepared in different buffers with the pH ranging from 1 to 11. As can be seen in Fig. 5, the amount

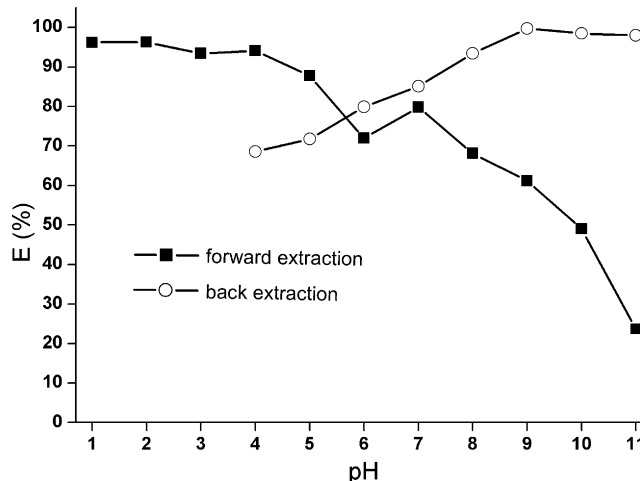


Fig. 5.

of lysozyme transferred to the IL phase decreased while its pH increased. When the pH of the buffer was decreased, the positive charges on the surface of lysozyme increased. This resulted in electrostatic interactions between the three negatively charged sulfonate groups on CB and the positive surface charge on lysozyme molecules. In addition to hydrophobic and electrostatic interactions, lysozyme was considered to form a more folded structure under pH 7 that would maximize lysozyme adsorption onto CB, reported in a study of dye–ligand adsorption [15]. The adsorption decreased while the pH was lower and higher than 7. However, the extraction in the present study was higher under pH lower than 7. In examination of amino acid extraction using [BMIM][PF<sub>6</sub>], the extraction was increased under pH lower than 7 [8]. A mechanism of ion exchange between BMIM<sup>+</sup> cations with amino acid cations was proposed. The BMIM<sup>+</sup> ions of [BMIM][PF<sub>6</sub>] were replaced by amino acid cations and transferred into the aqueous solution. The same process might also occur under lower pH buffers in which positively charged lysozyme molecules were exchanged with BMIM<sup>+</sup> in the IL phase.

Although the extraction was extremely high under pH 1–3, we decided to choose pH 4 for the following experiments to ensure that the protein molecules remained intact during the extraction. After forward extraction, the IL phase was transferred to the second buffer with pH ranging from 4 to 11. The stripping solution contained 1 M KCl, a high ionic strength, in order to carry out back extractions. Since the high salt concentration would change the equilibrium of dye–protein affinity adsorption [23], the desired proteins were recovered by increasing the ionic strength of the buffer. As the pH of the aqueous solution increased, the back extraction became greater. Lysozyme molecules were almost quantitatively recovered from the IL phase under pH 9–11. The lysozyme recovery of the back extraction was about 68% at pH 4, the same pH used in forward extraction.

#### 3.4. Lysozyme extraction using a reused IL extraction phase

One of the advantages of IL is it can be easily recycled. In the extraction phase, [BMIM]<sub>3</sub>[CB] in [BMIM][PF<sub>6</sub>] was reused to examine if it remained effective for lysozyme extraction. Forward extraction was conducted with the lysozyme sample (500 mg/l) buffered at pH 4, and back extraction was then performed with two different buffers of pH 8 and 11, containing also

1 M KCl. The protein concentrations in the aqueous solutions were determined to calculate the extraction efficiency. The used IL extraction solution was washed with an equal volume of 1 M KCl after each back extraction in order to remove the residual lysozyme, and then washed with an equal volume of de-ionized water. The procedures were repeated for 8 times to examine the extraction efficiencies. The resultant recoveries of lysozyme using back extraction under pH 11 after each complete forward and back cycle for all eight extractions ranged between 87% and 93%. Similarly, the recoveries of lysozyme at pH 8 stayed above 85% for all eight cycles. The results indicated that extractions of the forward and back processes remained essentially the same for all cycles.

#### 3.5. Extractions for other proteins

In addition to lysozyme, extractions for other proteins were tested to investigate the specificity of this CB dye modified compound. Protein samples of lysozyme, ovalbumin, BSA, and cytochrome *c* (500 mg/l each) were prepared in pH 7 and pH 11 buffers. Forward extractions were carried out for each solution for 3 times. The extraction results are listed in Table 1. In addition, blank measurements were performed using neat [BMIM][PF<sub>6</sub>] without adding [BMIM]<sub>3</sub>[CB].

Some lysozyme (4.4%) and cytochrome *c* (6.1%) molecules were transferred to the neat [BMIM][PF<sub>6</sub>] under pH 7, while BSA and ovalbumin molecules remained in the sample solutions. However, 81% lysozyme molecules transferred to the IL phase in the presence of [BMIM]<sub>3</sub>[CB], while the extraction of cytochrome *c* remained the same. This explained the selective affinity of [BMIM]<sub>3</sub>[CB] towards lysozyme. Similar to amino acids [24], both the lysozyme and cytochrome *c* molecules carried positive charges under pH 7, which interacted with the PF<sub>6</sub><sup>−</sup> moiety, thus tending to move to the IL phase. The anion of IL appeared to play an important role in the solubility and stability of cytochrome *c* in the IL [25], while the cationic part of the IL showed no significant effect on protein solubility. Under pH 11, none of the proteins were extracted into the neat [BMIM][PF<sub>6</sub>] phase. Only lysozyme was extracted into the IL phase in the presence of [BMIM]<sub>3</sub>[CB]. This again proved the specificity of [BMIM]<sub>3</sub>[CB] to lysozyme molecules. Lysozyme under pH 11 (close to its isoelectric point) did not carry a significant positive charge; therefore it was unfavorable for electrostatic interactions with the extraction phase. Accordingly, it resulted in lower extraction under pH 11 in contrast to pH 7.

Table 1  
Forward extractions for four proteins

IL extraction phase	Neat [BMIM][PF <sub>6</sub> ]	[BMIM] <sub>3</sub> [CB] in [BMIM][PF <sub>6</sub> ]	Neat [BMIM][PF <sub>6</sub> ]	[BMIM] <sub>3</sub> [CB] in [BMIM][PF <sub>6</sub> ]
Sample solution	pH 7	pH 7	pH 11	pH 11
Lysozyme	4.4 ± 1.1% <sup>a</sup>	81.2 ± 2.4%	–	23.6 ± 2.6%
Cytochrome <i>c</i>	6.1 ± 0.6%	6.2 ± 1.5%	–	–
Ovalbumin	–	–	–	–
BSA	–	–	–	–

–, not detectable.

<sup>a</sup> Average of 3 measurements ± S.D.

In addition to dye–ligand affinity [26,27], electrostatic repulsion between charged BSA and CB would increase while the pH was adjusted above 4.7, the *pI* of BSA. The BSA molecules tended to adsorb on CB-modified solid matrixes by hydrophobic interaction [13,27]. However, it showed no extraction under either pH 7 or pH 11 in this experiment. This might be due to the unfavorable solvation strength for BSA transferring into the IL phase that greatly weakened the CB-BSA affinity. Without dye–ligand interaction [13], there showed no sign of ovalbumin extraction in our study.

#### 4. Conclusions

In summary, imidazolium-based organic salt [BMIM]<sub>3</sub>[CB] with specific affinity interaction to lysozyme was synthesized by ion-exchange reaction. The extraction phase made by dissolving organic salt in the water-immiscible room-temperature IL [BMIM][PF<sub>6</sub>] was applied for the liquid–liquid extraction of lysozyme. Forward and back extractions of lysozyme were significantly affected by the pH and the ionic strength of the aqueous phase. In addition to affinity, electrostatic interactions also played a major role in the lysozyme transfer. Furthermore, the extraction phase can be reused without losing its extraction ability.

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