Enzymatic Synthesis of Dipeptides in Ionic Liquids

Sanjay V. Malhotra*,#, Chengdong Zhang and Hao Wang

Department of Chemistry and Environmental Science, New Jersey Institute of Technology, University Heights, Newark, NJ, 07010 USA

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Abstract: Immobilized protease-catalyzed synthesis of peptides from natural amino acids has been achieved in ionic liquids (ILs). Three ionic liquids BMIM.PF_6, BMIM.BF_4 and EtPy.BF_4 have been tested; an effective yet simple and practical method has been devised. The product yields are comparable with those seen in molecular solvents. A max yield of 49 % for Boc-Leu-TrpOEt has been obtained. This study shows that the advantages seen in organic solvents can also be achieved in ILs, and use of such medium could provide a viable alternative to organic and aqueous media for bio-catalysis.

Keywords: Enzymatic synthesis, protease, dipeptides, ionic liquid.

INTRODUCTION

Peptides represent new opportunities as drugs, diagnostics reagents, agricultural chemicals and even as additives in food [1-3]. Therefore, strong interest in the peptide area is not surprising, and development of methodologies for obtaining peptides in high yields, good purity and in large scale production is a field of growing interest. In water, the equilibrium of amide bond formation and hydrolysis is generally shifted towards hydrolysis, and thus enzyme naturally hydrolyzes amide bonds. To overcome this, there have been advances in the use of non-aqueous media which have made significant effect on bio-transformations of interest to chemical and pharmaceutical industry [4, 5]. It is now well accepted that organic solvents offer advantages over aqueous medium mainly in thermodynamically unfavored reactions [4]. However, despite these advances organic medium suffers from key drawbacks, such as poor solubility of charged and polar compounds of high volatility. These limitations have prompted the design of new reaction media which are non-aqueous yet help to overcome the drawbacks of organic solvents. Notable example is the ionic liquid, a new class of compounds having unique physiochemical properties, and therefore, studied for various organic transformations, e.g. hydrogenation, oxidation, substitution, Heck reaction etc. [6-9]. Since, it is reported earlier that protease has good stability in alcoholic medium [10], it can catalyze nucleophilic reactions of both D- & L- amino acids and has been used in the amidation reactions [11-16]; we decided to study dipeptide synthesis by using Protease immobilized on Celite. Use of immobilized enzyme would facilitate the purification and allows an easy recycling of the enzymatic catalyst. Also, in place of aqueous and organic media, we employed hydrophobic IL i.e. BMIM.PF_6, and hydrophilic ILs i.e. BMIM.BF_4 and EtPy.BF_4. The work presented in this paper is a new approach to the enzymatic synthesis of dipeptides.

*Address correspondence to this author at the Department of Chemistry and Environmental Science, New Jersey Institute of Technology, University Heights, Newark, NJ, 07010 USA; Tel: +1 301 846 5141; Fax: +1 301 846 5206; E-mail: malhotrasa@mail.nih.gov

#Current address: Laboratory of Synthetic Chemistry, SAIC-Frederick Inc., National Cancer Institute at Frederick, 1050 Boyles Street, Frederick, Maryland 21702.

RESULTS AND DISCUSSION

Earlier we reported the use of ionic liquid EtPy.TFA for the first time as a catalyst for esterification of amino acids, including unnatural compounds [17]. After N-acetylation, the N-protected amino acid was dissolved in anhydrous ethyl or isopropyl alcohol followed by adding the ionic liquid. Yields were generally good, especially for ethyl esters. These promising results demonstrated the potential of ionic liquids for amino acid chemistry, more specifically the peptide coupling. Motivated by these results, we started to examine construction of peptide bonds in ionic media by means of enzymes. Enzymatic reactions in ionic liquids have been reported earlier [18]. The methods require additional steps of separation and cleaning of enzymes before next use. To simplify this process, enzyme in our study was first immobilized on celite. It was then used repeatedly for synthesis of peptides by using a host of amino acids shown in Fig. (**1**).

In a typical reaction, (see experimental procedure) first an N-protected amino acid was treated with immobilized protease resulting in the enzyme coupled intermediate. Subsequent reaction of the intermediate with C-protected amino acid gives product dipeptide. A general reaction scheme is shown in Fig. (**2**).

Results of various peptide syntheses are shown in Table **1**. As can be seen in Fig. (**2**), once the intermediate is formed there are two competitive reactions i.e. with water and/or with C-protected amino acid. Higher yields of products would be obtained if the reaction with second amino acid is faster than that with water. Therefore, the nature of reaction medium could play an important role. For testing, we carried out a number of syntheses in hydrophobic ionic liquid

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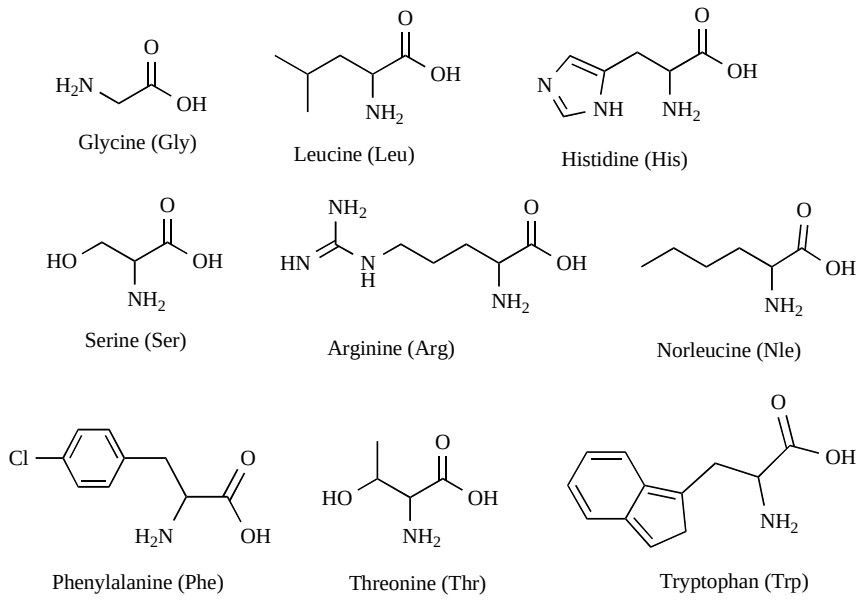

Fig. (1). Structures of amino acids studied for peptide synthesis.

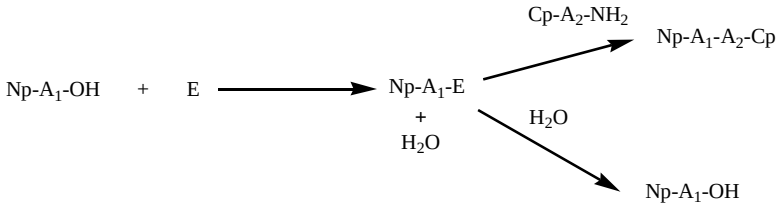

Fig. (2). Enzymatic synthesis of dipeptide (Np: N-terminal protected amino acid, Cp-C-terminal protected amino acid, and E-protease).

Table 1. Enzymatic Synthesis of Dipeptides in Ionic Liquids (37°C, 5 % Water Content, 48 h)

Serial #	Amino Acid-1	Amino Acid-2	Peptide	Ionic Liquid	Yield (%)
1.	Boc-Leu	TrpOEt	Boc-Leu-TrpOEt	BMIM.PF_6	49
2.	Boc-Leu	GlyOEt	Boc-Leu-GlyOEt	BMIM.PF_6	22
3.	Boc-Leu	GlyOEt	Boc-Leu-GlyOEt	EtPy.BF_4	40
4.	Boc-Leu	SerOMe	Boc-Leu-SerOMe	BMIM.PF_6	38
5.	Boc-Leu	SerOMe	Boc-Leu-SerOMe	BMIM.BF_4	40
6.	Boc-Gly	TrpOEt	Boc-Gly-TrpOEt	BMIM.PF_6	19
7.	Boc-Gly	TrpOEt	Boc-Gly-TrpOEt	EtPy.BF_4	26
8.	Boc-Leu	Phe(Cl)OEt	Boc-Leu-Phe(Cl)OEt	BMIM.PF_6	45
9.	Boc-Arg	SerOEt	Boc-Arg-SerOEt	BMIM.PF_6	12
10.	Boc-Arg	NleOMe	Boc-Arg-NleOMe	BMIM.PF_6	36
11.	Boc-Arg	Phe(Cl)OEt	Boc-Arg-Phe(Cl)OEt	BMIM.PF_6	7
12.	Boc-His	SerOMe	Boc-His-SerOMe	BMIM.BF_4	25
13.	Boc-His	TrpOEt	Boc-His-TrpOEt	EtPy.BF_4	31
14.	Boc-His	ThrOMe	Boc-His-ThrOMe	EtPy.BF_4	18
15.	Boc-His	Phe(Cl)OEt	Boc-His-Phe(Cl)OEt	EtPy.BF_4	44

BMIM.PF_3 and also in hydrophilic ILs i.e. BMIM.BF_4 and EtPy.BF_4.

As data show the solubility of amino acid-2 in solvent affects the peptide yield. For example, high yield of Boc-Leu-TrpOEt is obtained in BMIM.PF_6 while in the same IL as solvent lower yield of Boc-Leu-GlyOEt is seen (entry 2). This is because GlyOEt has low solubility in BMIM.PF_6. When same reaction is carried out in a hydrophilic IL EtPy.BF_4 the yield is significantly higher. Similarly, a comparison can also be seen in the synthesis of Boc-Leu-

Table 2. Comparison Enzymatic Reaction in Ionic Liquid and Organic Solvent (37 ^{o}C, 5 % Water Content, 48 h)

Peptides	Yield (%) (Ionic Liquid)	Yield (%) (Organic Solvent)
Boc-Leu-TrpOEt	49 ($BMIM.PF_6$)	21 (Acetone)
Boc-Leu-SerOMe	38 ($BMIM.PF_6$)	30 (Acetone)
Boc-Leu-Phe(Cl)OEt	45 ($EtPy.BF_4$)	55 (Ethyl Acetate)
Boc-His-TrpOEt	31 ($EtPy.BF_4$]	64 (Ethyl Acetate)
Boc-Gly-TrpOEt	26 ($EtPy.BF_4$)	7 (Acetone)

SerOMe (entry 4 and 5) and Boc-Gly-TrpOEt (entry 6 and 7).

Since, protease has also been by used in the peptide synthesis using organic solvent [1], we decided to compare the synthesis of few representative dipeptides in both ionic liquids and organic solvents. Results are shown in Table **2**.

Earlier, one report suggested the possibility of peptide synthesis in anhydrous ethanol [15], however, in our hand, reaction did not occur when ethanol was used as solvent. We believe this is due to the denaturalization of enzyme and lose of its catalytic activity. Also, the polarity of ionic liquids can be tuned as opposed to the fixed polarity of known molecular solvents, which suggests the possibility of enzymatic reactions using ILs, where the most polar solvents are ineffective. This could extend many enzyme catalyzed reactions to a wide polarity range. Further, it should be noted that mass spectrometric analysis of the solvent extraction showed the presence of dipeptide product which could account for lower yields. This observation is similar to earlier report on peptide synthesis [19]. Overall yields in our study in ionic liquid medium are comparable to those reported in organic medium [15]. Lastly, reusability of ionic liquid was tested with recycled $BMIM.PF_6$ for the synthesis of Boc-Leu-TrpOEt and yield of 48 %, 42% and 41 % were obtained for three consecutive runs. This further establishes the potential of ILs for peptide synthesis.

CONCLUSIONS

Our study has demonstrated the utility of ionic liquids in the synthesis of peptide catalyzed by immobilized protease. Both hydrophobic and hydrophilic ILs are found to be effective for this reaction. Results also suggest that both organic solvents and ionic liquids media could be useful for peptide synthesis. However, systematic investigation should be done to find the most suitable medium for large scale synthesis.

EXPERIMENTAL

General

Materials and reagents were of highest commercially available grades and used as obtained. Phosphate buffer (pH 7.2), triethylamine, anhydrous ethanol, toluene, ethyl acetate, acetone, and Celite were from Aldrich. Protease (alcalase from Bacillus Licheniformis) was from Sigma Company. All Boc- protected amino acids and amino acid ester were purchased from Sigma Company. LC-MS analysis was carried out by HPLC (waters) system at detection of 254 nm with flow rate of 1.0ml/min. The mobile phase consisted of acetonitrile/water (50/50 % v/v).The column was Alltech C18 5µm, 4.6x250mm. All the products were confirmed by mass spectroscopy (LCQ Advantage). ^{1}H NMR analysis was performed on Varian 300 MHz instrument.

Ionic Liquid Preparation

Synthesis of ($EtPy.BF_4$)

$EtPy.BF_4$ was synthesized according to Zhao's method [9] and characterized by NMR and FTIR.

A general synthetic procedure could be described as follows: Tetrafluoroboric acid (0.2mol) was slowly added to stirred slurry of silver (I) oxide (0.1 mol) and distilled water (50 ml). To avoid photodegradation of silver (I) oxide, the reaction mixture was fully covered with aluminum foil. The reaction mixture was stirred continuously until the reaction was complete, which was indicated by the formation of a solution. A solution of N-ethyl-pyridinium bromide (0.2 mol) was added to the reaction mixture. As reaction took place and ILs formed, a yellow precipitate of silver (I) bromide started to be observed. The mixture was stirred at room temperature until no precipitate formed. The precipitate of silver (I) bromide was filtered off, and then the solvent was removed by rotary evaporation under vacuum at about 65 °C. The resulting ionic liquids were put in an oven overnight at 65 °C to remove the moisture.

Synthesis of $BMIM.BF_4$

Tetrafluoroboric acid (13.3 ml, 0.173mol) was slowly added to stir slurry of silver (I) oxide (20.0 g, 0.0863 mol) in 50 ml distilled water over 10 minutes. To avoid photo degradation of Ag_2O, the reaction mixture was covered with aluminum foil. Until the Ag_2O was fully reacted, a solution of BMIM.Cl (30.15 g, 0.1726 mol) in 150 ml distilled water was added to the reaction mixture and stirred at room temperature for 3 h. The yellow precipitate was filtered off,

and solvent was removed at 65 ^{o}C under vacuum. Yield is 97%.

Purification of Ionic Liquids

The ionic liquids were purified according to the literature procedure [20] with following modification. Ionic liquid was re-dissolved in acetonitrile or methanol and any excess of Br^- or Cl^- was removed by adding silver nitrate drop by drop till no more halide was left. Neutral alumina was added to the solution, and stirred over night to absorb un-reacted trace imidazole or pyridinium reactant. After evaporation under vacuum, clear ionic liquid product was obtained.

Recycling of Ionic Liquid

Immobilized enzyme was filtered off after the reaction, BMIM.PF_6 was washed by 5% citric acid, $NaHCO_3$ followed by water and the dried under vacuum at 60^{o}C. For hydrophilic ionic liquids EtPy.BF_4 and BMIM.BF_4, after separating enzyme, 10ml methanol was added with small amount of neutral alumina, shaking over night. After evaporating, purified ionic liquid was dried under vacuum for reuse.

Preparation of Immobilized Protease

Enzyme-Celite was prepared according to reported procedure [1]. A 2 ml aqueous solution of protease (15 units/mg) was added to a mixture of 2 g Celite and5 ml buffer solution (pH 7). It was mixed thoroughly and then dried overnight under vacuum at room temperature.

Representative Procedure for Enzymatic Synthesis

The N-Boc protected acyl alcohol (1 equiv.) and nucleophile (1.5 equiv.) were taken in 5 ml ionic liquid. When the amine component was in hydrochloride form, equal amount of triethylamine was added. 5% (v/v) buffer was added as water content. The reaction was started by adding 1g immobilized enzyme. After being shaken at 37°C for 48 hours, 20 ml toluene was used to extract product from ionic liquid continuously over two days. The extract solution was washed with 5 % citric acid, followed by water and 5 % $NaHCO_3$. After the toluene was evaporated under vacuum, the residue was dissolved in 2ml methanol. The reaction progress was monitored by thin-layer chromatographic analysis performed on a silica gel pre-coated plate in ethyl acetate liquid phase. Amino acids were tested by ninhydrin.

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