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Organic-phase biological buffers for biochemical and biological research in organic media

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ABSTRACT

A long-standing problem in enzymatic catalysis in organic media is finding a buffer that keeps a constant pH, as there are no commercially available biological buffers for such media. The biological buffers such as Good's buffers that are commonly used in aqueous media are insoluble in almost all organic solvents, and there is nowadays a pressing need for a buffer for organic media. Good's buffers were designed based on sets of criteria which make them extensively used in biochemical and biological research. In this context, we show that when Good's buffers turned into ionic liquids, so-called Good's buffer ionic liquids (GB-ILs), they became highly soluble in polar organic media, such as methanol and ethanol. This implies that a huge number of organo-soluble buffers are expected to be commercially available for such solvents. Twenty five GB-ILs (tetramethylammonium, tetraethylammonium, tetrabutylammonium, 1-ethyl-3-methy limidazolium, and choline salts of Tricine, TES, MES, HEPES, and CHES) were tested as organic-phase buffers for pure methanol, and their buffer capacities in methanol were found to be very close to that in water. A series of mixed GB-ILs was also formulated as universal buffers in methanol. Conductor-like screening model for real solvents (COSMO-RS) calculations were performed to show why GB-ILs gained high solubility in methanol.

1. Introduction

Non-aqueous enzymology is becoming increasingly important for applications in biotechnological processes. It is of importance when the reactants are poorly soluble in aqueous media, which certainly speeds up reaction rates [1]. It has the ability to catalyze reactions that are kinetically or thermodynamically unfavorable in water, e.g. transesterification, thioesterification, and aminolysis [1]. Transesterification is a chemical process of exchanging acyl groups between an ester and a monohydric alcohol, to produce biodiesel (monoalkyl esters) and glycerine [2]. Biodiesel produced by the transesterification of oils or animal fats is a promising alternative diesel fuel regarding the limited resources of fossil fuel and the environmental impacts [2]. There are many monohydric alcohols used for biodiesel production, such as methanol, ethanol, propanol, 2-opropanol, and butanol. Enzyme-catalyzed transesterification, especially those using lipase, for the production of biodiesel allow using alcohol in a solvent free system [2].

Most enzymes are, however, less active in organic solvents or in low-water concentration media and they must be stabilized and protected from inactivation. An essential issue in this context is that of enzyme ionization, which is a key determinant of its activity. The enzyme can have its activity changed significantly by adding or removing protons. The catalytic activity of enzymes in both aqueous and organic media is strongly affected by the pH displaying an optimum pH. The pH effect upon the enzymatic activity can be gauged from examples such as the subtilisin (cross-linked crystals) that can be activated more than 100-fold in pentanone by adding a "buffer" [3], or the report that the activity of enzymes in organic solvents is highly dependent on the pH of the last aqueous solution on which the enzyme was lyophilized (pH memory phenomenon) [3], the protonation state of the enzyme's ionisable groups being retained in the organic solvent. The ionization state of an enzyme in organic solvents can thus be manipulated by lyophilization of the enzyme from an aqueous buffered solution. This is an unpractical and expensive approach that can be thwarted since the pH memory can be altered if acidic or alkaline species are used in the reaction mixture [4–6]. The use of buffers soluble in organic phases to control the pH of a non-aqueous medium would be a more adequate approach. Unfortunately, most known buffers are insoluble in organic solvents due to their charged nature and high polarity, being the development of alternative buffers for such organic media a pressing need.

Buffers for organic phases can be formulated from hydrophobic bases or acids and their salts, such as combinations of triisooctylamine with its hydrochloride, or of triphenylacetic acid with its sodium salt [7,8]. Dolman et al. synthesized more hydrophobic buffers based on functionalized, dendritic polybenzylethers [9]. These organo-soluble buffers, although limited in number, effectively erase the enzyme's pH

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memory and control its protonation state [10]. Solid-state acid–base buffers have been also proposed for use in organic media [10–14]. These solid buffers consist of a zwitterionic compound (Good's buffers among the others) and its sodium salt. Although these buffers were able to control the ionization state of the catalytic triad of subtilisin in organic solvents [13], they failed with papain [10].

Recently, ionic liquids with buffering characteristics has been synthesized [15–17]. Room temperature ionic liquids (RTILs) are normally described as molten salts which are liquid at room temperature, or salts melting below 100 °C. The ILs have enhanced the enzyme activity and stability in comparison to other solvent media [18,19]. Very limited attention, however, has been devoted to prepare ILs that can control the pH of non-aqueous systems. Yuan and co-workers [15–17] have synthesized buffer-like ILs by neutralization of imidazolium hydroxides with polybasic acids such as the phthalic, tartaric, and phosphoric acid. These ILs are miscible with polar solvents such as methanol, dichloromethane, and dimethylformamide (DMF). MacFarlane et al. [20] have described that hydrated ILs, such as choline dihydrogenphosphate have buffering action. Although these buffer-like ILs have good solubility in the above-mentioned organic solvents, but their anions are not inert for one reason or another. For example, using phosphate ions precipitates metal ions such as, calcium, zinc, or magnesium, metals that are essential to maintain the biological function of some enzymes [21,22].

Most biological buffers used today were developed by Good and his colleagues [21,22] and are known as Good's buffers (GBs). Their acronyms are spread in literature and laboratory manuals, and have become new words; i.e., HEPES buffer appears as the word Hepes. These buffers are zwitterionic amino acids, either *N*-substituted taurine or glycine derivatives, which provide good coverage of the physiological pH range (6.1–10.4). Good set forth several criteria to define the best buffers for biochemical systems, his buffers fulfilling most of these criteria: The buffers should be chemically inert; non-toxic; present no absorption in the UV–visible region; no formation of complexes with metal ions; be easy to prepare and inexpensive; and their pKa values should not vary with temperature. They should also ideally have high water solubility and low lipid solubility so that they do not permeate the cell membranes. Good's buffers are however highly soluble in water but insoluble in pure organic solvents such as aliphatic alcohols, cyclic ethers, acetone, and acetonitrile [23,24]. They cannot thus be used for controlling the pH in organic solvents. The high affinity of GBs to water molecules is due to their high polarity [23,24].

We have recently introduced a new class of buffer-like ILs, Good's buffer ionic liquids (GB-ILs), which derived from Good's buffers (e.g., TES, TAPS, TAPSO, HEPES, EPPS, MES, MOPS, MOPSO, CHES, CAPS, CAPSO, BES, Bicine, and Tricine) as anions and several cations such as tetramethylammonium ($[N_{1111}]^+$), tetraethylammonium ($[N_{2222}]^+$), tetrabutylammonium ($[N_{4444}]^+$), 1-ethyl-3-methy limidazolium ($[C_2mim]^+$), and cholinium ($[Ch]^+$) [25–29]. These GB-ILs have proven to be remarkably effective as biological buffers and protein stabilizers in aqueous solution [25–30]. Some of these GB-ILs were also found to form aqueous biphasic systems (ABS) when mixed with aqueous solutions of inorganic salts, organic salts, or PPG 400 (poly(propylene)glycol with a molecular weight of 400 g mol^{-1}), and were successfully used to extract biomolecules such as protein and antibody [25–30] without adding external buffers. Here, we have observed for the first time that GB-ILs, unlike common GB, are highly soluble in polar organic solvents, e.g. methanol and ethanol. The goal of this work is to investigate whether GB-ILs could still act as buffering agents in these organic solvents. The presence of the buffering action of GB-ILs is confirmed by measuring their pH-profiles in methanol as an example. The reason of choosing methanol is the most widely used alcohol for biodiesel production using enzymatic transesterification process. The reasons behind the high solubility of GB-ILs in methanol, as compared to the conventional Good buffers, are explained theoretically by conductor-like screening model for real solvents (COSMO-RS) calculations.

2. Materials and methods

2.1. Materials

The buffers, MES (purity >99 wt%), TES (purity >99 wt%), HEPES (purity >99.5 wt%), Tricine (purity >99 wt%), and CHES (purity >99 wt%), were obtained from Sigma–Aldrich Chemical Co. The hydroxides, tetramethylammonium (25 wt% in H_2O), tetraethylammonium (25 wt% in H_2O), tetrabutylammonium (40 wt% in H_2O), and 1-ethyl-3-methylimidazolium (10 wt% in H_2O), were also provided by Sigma–Aldrich Chemical Co. (USA). Sodium hydroxide pellets obtained from Eka Chemicals, and 0.05 M HCl-methanol solution was prepared from 0.5 M HCl-methanol solution obtained from Sigma–Aldrich Chemical Co. (USA). Methanol (HPLC grade, purity >99.9%) was purchased from Fisher Scientific and acetonitrile (purity >99.7) was supplied from Lab-Scan. Purified water was obtained by a Milli-Q plus 185 water purifying system.

2.2. Synthesis and characterization of Good's buffer ionic liquids

The GB-ILs, $[C_2mim][GB]$, $[N_{1111}][GB]$, $[N_{2222}][GB]$, $[N_{4444}][GB]$, and [Ch][GB], were synthesized by neutralizing a slight excess of equimolar GB with the aqueous corresponding organic hydroxide aqueous solution as described in our earlier papers [24,25], where GB = MES, Tricine, TES, HEPES, and CHES. Brefiely, an aqueous solution of the organic hydroxide was added dropwise to a slightly excess equimolar buffer aqueous solution. The solution was stirred magnetically at ambient conditions for about 12 h. The reaction mixture was evaporated at 60 °C under vacuum using a rotary evaporator to obtain a viscous liquid. Then, a mixture of acetonitrile and methanol (1:1) was added to the viscous liquid and stirred vigorously at room temperature for 1 h to precipitate the unreacted buffer. The solution was then filtered to remove any precipitate presented. The synthesized GB-ILs were purified by washing with acetone several times. The solvent mixture was evaporated and GB-IL product was then dried under vacuum for 3 days at room temperature. The water content of GB-ILs was measured by Karl–Fischer coulometer (Metrohm Ltd., model 831) and it was found to be less than 0.05 wt%. The chemical structures of the GB-ILs were confirmed, respectively, by ^1H and ^{13}C NMR spectroscopy (Bruker AMX 300) operating at 300.13 and 75.47 MHz, and presented in our earlier papers [25,26]. The chemical structures of GB-IL are shown in Fig. 1.

2.3. Potentiometric titrations

pH titration profiles were performed in double-walled glass vessel using an automatic titrator (Metrohm 672) equipped with a dosimat 655 and a solvotrode (Metrohm 6.0229.100) for pH measurements in non-aqueous acid-base. The pH electrode was calibrated in aqueous solution with two standard buffers of pH 4.0 and 7.0. The temperature of the titration vessel was controlled at 298.2 ± 0.1 K by thermostatic water bath with continuous magnetic stirring. For measuring the pH profiles of GBs in water, 10 mL of 0.05 M GB was freshly prepared in water and titrated with 0.05 M NaOH. The pH profiles of GB-ILs in pure methanol were carried out by titrating 10 mL of 0.05 M GB-ILs prepared in methanol with 0.05 M NaOH/HCl dissolved in methanol. At least two repeated measurements were performed for each pH profile.

Since the pH meter is calibrated using in aqueous solution, the $pH_{methanol}$ reading obtained in non-aqueous medium differs by an amount, δ, from the pH_{water} reading obtained when the pH meter standardized using aqueous buffer solution. The δ value for pure methanol is well known (−2.24 in the molarity scale), and, therefore, experimental pH_{water} allow to get the $pH_{methanol}$, by means of $pH_{methanol} = pH_{water} + 2.24$ [31–35].