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# Efficient separation of mannan–protein mixtures by ionic liquid aqueous two-phase system, comparison with lectin affinity purification

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ABSTRACT

Analysis of carbohydrates from complex biological samples often requires their isolation from proteins and other contaminants to avoid interference. An effective separation of mannan–protein mixtures by 1-butyl-3-methylimidazolium bromide/$K_2HPO_4$ ionic liquid aqueous two-phase system (IL-APTS) is reported. Extraction efficiency of bovine serum albumin (BSA) ranged from 92% to 97% while extraction efficiency of mannan reached values from 95% to about 100% depending on phase and/or model sample composition. On the contrary, lower efficiency of BSA removal (73–84%) was recorded for lectin affinity purification with concanavalin A–triazine bead cellulose (Con A-TBC); the low mannan-binding capacity was limiting factor here. The size exclusion chromatography pattern of model mannan–BSA samples after both IL-APTS and Con A-TBC treatments were consistent with the spectrophotometric component analysis. In case of biological experiment, the ionic liquid separation technique was superior in pre-purification of 2-aminobenzamide-labelled mannan from cell culture medium prior to HPLC-FLD analysis.


## 1. Introduction

Carbohydrates and proteins are essential biological macromolecules of all living organisms. They generally coexist together performing various functions in biological systems. For structural, functional or quantitative analysis, it is often necessary to separate carbohydrates from proteins or vice versa. The separation is necessary especially in case of such analytical methods where carbohydrates and proteins could interfere, e.g. fluorescence detection (FLD) of labelled carbohydrates [1] or Bradford protein assay [2]. Several methods have been applied to separation of polysaccharides from polysaccharide–protein samples. Differential precipitation of proteins was used to isolate polysaccharide fraction from *Pseudomonas fluorescens* biofilm extracts in order to obtain standard of high purity for sugar analysis [3]. Optimised hollow fibre membrane ultrafiltration was applied to separation of protein and polysaccharide from spent brewer's yeast extracts [4]. New solid-phase extraction cartridge developed especially for high molecular mass polysaccharides recovery was introduced and applied in *Lactobacillus* KLB58 polysaccharide sample pre-treatment prior to composition analysis [5]. Combination of ultrafiltration with liquid–liquid extraction by traditional poly(ethylene glycol) based aqueous two-phase system (ATPS) was reported for purification of polysaccharides from the *Aloe varavia* pulp [6].

Besides classic polymeric and micellar ATPSs [7,8], hydrophilic ionic liquids (IL), as environmentally-friendly designer solvents, are also capable of forming ATPS in the presence of water-structuring salt [9]. This type of IL-based ATPS (IL-ATPS) has been successfully applied to separation, concentration, and purification of proteins, antibiotics, antioxidants, alkanoids, drugs, and small organic molecules [10]. However, selective separation of polysaccharides from polysaccharide–protein mixtures is rarely studied. The first example of selective separation of mono-, oligo- or polysaccharide model mixtures with BSA was reported by Pei et al. [11]. Similar model samples were used to test ATPS based on a novel ether-functionalized IL by the same group [12]. Besides these model studies, IL-ATPS was employed in simultaneous single-step extraction and purification of polysaccharides and proteins from *Aloe vera* [13] and *Cordyceps sinensis* [14].

Affinity purification using immobilized lectins, specific carbohydrate-binding proteins, is another approach in separation and purification of carbohydrates from complex biological mixtures [15,16]. Concanavalin A (Con A), lectin extracted from

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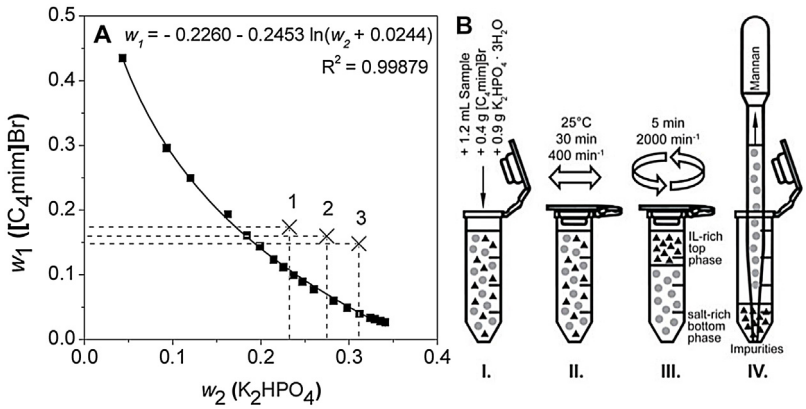


**Fig. 1.** Binodal curve of [$C_4$mim]Br/$K_2HPO_4$ aqueous two-phase system (IL-ATPS) at 25 °C adapted from [26]; 1–3 represent three chosen compositions differing in content of $K_2HPO_4$ (A). Liquid–liquid extraction by IL-ATPS includes four steps: ingredients mixing (I), thermo-shaking (II), centrifuging (III), and purified sample siphoning off (IV) (B).

*Canavalia ensiformis*, is one of the most commonly used lectins. It demonstrates binding specificity towards carbohydrate residues containing internal or non-reducing terminal α-D-glucopyranosyl or α-D-mannopyranosyl moieties [17]. Though mainly used in glycoconjugate or glycoconjugate-derived oligosaccharide isolation, it has been successfully applied also for purification or removal of polysaccharides [18–21].

Here we present an efficient separation of ultrasonically degraded mannan from *Candida albicans* [22] from protein contamination by liquid–liquid extraction with IL-ATPS consisting of 1-butyl-3-methylimidazolium bromide ([$C_4$mim]Br) and $K_2HPO_4$. An aqueous mixture of mannan and bovine serum albumin (BSA) was used as a model. Information gained from the model experiments were applied in biological experiment for isolation of 2-aminobenzamide (2AB) labelled mannan [23] from cell culture medium prior to HPLC-FLD analysis. Commonly used affinity purification by Con A immobilized on chlorotriazine bead cellulose (Con A-TBC) was employed for comparison.

## 2. Material and methods

### 2.1. Material

Ultrasonically degraded (≈35 kDa, PDI = 1.6) [22] and 2AB-labelled (≈28 kDa, 1.4 mass% 2AB) [23] mannan from *Candida albicans* serotype A (CCY 29-3-100) were used. Bovine serum albumin (BSA; Sigma–Aldrich, Germany) was used as a model protein. 1-Butyl-3-methylimidazolium bromide ([$C_4$mim]Br; > 97.0 mass%) and cyanuric chloride (2,4,6-trichloro-1,3,5-triazine) were from Sigma–Aldrich (Germany). Beaded cellulose Perlose MT 100 (100–250 μm) was purchased from SChZ (Czech Republic) and lectin of *Canavalia ensiformis* (Concanavalin A) from Serva (Germany). All other chemicals were of reagent grade and used without further purification.

### 2.2. Mannan–BSA model separation

#### 2.2.1. Ionic liquid based aqueous two-phase system

IL-ATPSs were prepared by mixing 0.4 g of [$C_4$mim]Br; 0.7 g, 0.9 g or 1.1 g of $K_2HPO_4 \cdot 3H_2O$ (Fig. 1A); and 1.2 mL of aq. solution of mannan (1 mg/mL) and BSA (4 mg/mL, 8 mg/mL or 16 mg/mL) in graduated vials. Another mixture with the same phase components but without mannan and BSA was prepared as a blank. Samples and blanks were shaken for 30 min at 25 °C and speed of 400 min$^{-1}$ (Microplate Incubator Shaker PST-60 HL plus, Boeco, Germany) and subsequently centrifuged for 5 min at 2000 min$^{-1}$ in order to ensure complete phase separation (Universal 320R centrifuge, Hettich, Germany). The volumes of top and bottom phases were recorded and the bottom phase was siphoned off (Fig. 1B).

Three 10 μL samples of bottom phase and three 60 μL samples of each phase were diluted with water to 500 μL for sugar and protein analysis, respectively. The concentration of mannan was determined spectrophotometrically by phenol–sulfuric acid assay measuring absorbance at 490 nm. Standard aq. solution of mannose (1 mg/mL) was used for calibration. The BSA content was determined spectrophotometrically measuring absorbance at 280 nm. Standard solution of BSA (4 mg/mL) was used for calibration. All measurements were performed using UV mini 1240 UV–vis spectrophotometer (Shimadzu, Japan) against corresponding blank experiment. Extraction efficiency of mannan ($E_{Man}$) was calculated using the following equation [12]:

$$E_{Man} = 100 c_b V_b / c_0 V_0 \quad (1)$$

where $c_b$ is equilibrium concentration of the partitioned mannan in the salt-rich bottom phase, $V_b$ is volume of bottom phase, $c_0$ and $V_0$ represents initial concentration and volume of mannan solution. Extraction efficiency of BSA ($E_{BSA}$) was calculated using the following equation [12]:

$$E_{BSA} = 100 c_t V_t / (c_t V_t + c_b V_b) \quad (2)$$

where $c_t$ and $c_b$ are equilibrium concentrations of the partitioned BSA in the IL-rich top phase and salt-rich bottom phase, $V_t$ and $V_b$ stand for volumes of the top and bottom phase, respectively.

#### 2.2.2. Conanavalin A–triazine bead cellulose affinity purification

Concanavalin A immobilized on chlorotriazine bead cellulose (Con A-TBC) was prepared according to procedure described elsewhere in detail [24]. Solution (3 mL) of mannan (1 mg, 2 mg or 4 mg) and BSA (4 mg) in 50 mM acetate buffer (pH 4.7; with 0.1 M NaCl, 0.1 mM $Ca^{2+}$ and 0.1 mM $Mn^{2+}$) was incubated with 5 mL (6 g of wet mass) Con A-TBC overnight at ambient temperature. Unbound mannan and BSA were washed out by the same buffer (10 × 3 mL). Methyl α-D-mannopyranoside (0.1 M) in 50 mM acetate buffer (pH 4.7; with 0.1 M NaCl) was used for mannan release (4 × 3 mL).

The concentration of mannan and BSA was determined spectrophotometrically as described in section 2.2.1, without dilution of samples. Mannan-binding capacity ($C$) of Con A-TBC was determined as a difference between the initial amount of mannan in sample and the amount of unbound mannan. Efficiency of BSA removal ($E$) was calculated using the following equation:

$$E = 100(1 - c/c_0) \quad (3)$$