**Enzymatically-boosted ionic liquid gas separation membranes using carbonic
anhydrase of biomass origin**

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Abstract

Nowadays there is a huge demand for new and sustainable technologies aiming the reduction of the greenhouse gas, in particular carbon dioxide emission. In this work, *enzymatically-boosted supported ionic liquid membrane* (EB-SILM) was developed to permeate carbon dioxide with improved efficiency. Firstly, the selected biocatalyst, *carbonic anhydrase* (CA) was prepared and purified from spinach, a cheap plant biomass containing the enzyme of our interest. Afterwards, the CA enzyme preparation was used for SILM fabrication in order to test the properties towards enhanced carbon dioxide permeation over CH₄, H₂ and N₂. The results indicate basically that EB-SILMs possess an increased ability to permeate CO₂ in comparison with enzymeless controls and therefore, may be viewed as a promising approach e.g. towards enhanced CO₂-capture bioprocesses.

Keywords: carbonic anhydrase, enzyme, ionic liquid, membrane, gas separation, CO₂ capture

1. Introduction

Reducing carbon emissions is an urgent task [1], where membranes could play an important role [2]. Among them, those made with ionic liquids (ILs) are potential candidates for the selective removal of CO₂ from gaseous mixtures [3-5]. Recently, the significant CO₂ absorption capacity of ILs – consisting of imidazolium-cation (C_nmim) and [Tf₂N]-anion – was confirmed [6,7]. Additionally, a specific enzyme, called *carbonic anhydrase* (CA) (E.C. 4.2.1.1.) was introduced as a promising option to develop biological CCS method [8]. CA is able to catalyze the reversible hydration of CO₂ [9]:

Moreover, Neves et al. [10] reported that the performance of supported ionic liquid membranes made of imidazolium-based IL with [Tf₂N]-anion could be improved by CA addition. However, to our knowledge, this *membrane-ionic liquid-enzyme* system was studied only by applying highly-purified, commercially available CA, which is extremely expensive since blood is mainly used as its source in health care applications. Nevertheless, CA can also be found in cheaper resources such as green plants and some works already demonstrated recovery of CA from biomass [11,12]. Therefore, in this research it was aimed to (i) develop a method for CA enzyme preparation from plant origin and (ii) use it in SILM – incorporating [bmim][Tf₂N] as a model IL – to take at least one further step towards CO₂ separation from gaseous effluents and more attractive bioprocesses.

2. Materials and Methods

2.1. Preparation of carbonic anhydrase enzyme

CA enzyme was prepared from fresh spinach leaves (*Spinacia oleracea*) bought from local market (stored at -20 °C until use). The other compounds used were Tris-HCl (Calbiochem) buffer, ethanol, NaOH and ammonium sulphate (Reanal, Hungary) having analytical purity.

Firstly, 400 g spinach leaves were pulled to pieces and put in a kitchen blender. In the device, the biomass was mixed with 96 (m/m)% ethanol (1 mL/g spinach) and chopped (500 W, 5 min). When shredding was done, vacuum filtration (FT-3-104-150 quantitative filter paper, Sartorius AG) was used to remove liquids. Thereafter, the remaining solid fraction (the filtration cake) was transferred to a beaker, fresh alcohol was added (same amount as for chopping) and the mixture was stirred (150 rpm) for 20 min at room temperature (23 ± 2 °C). As the time expired, the mixture was vacuum filtered again. This solid-liquid extraction was repeated for 5 cycles during which alcohol-soluble compounds e.g. pigments, oils, etc. were separated, meanwhile the proteins released after cell disruption (including CA) were aggregated with the cell debris in a denatured form. The alcohol fractions removed after the cycles were collected and regenerated.

In the following stage of downstream, the pulpy fraction was soaked in distilled water (1 mL/g spinach) for 12 hours at 4 °C. Subsequently, the liquid phase was taken and centrifuged (12000 rpm, 20 minutes). After that, the supernatant (containing our enzyme of interest) was dried at 40 °C under vacuum by a Heidolph VV2000 Rotadest. The obtained solid residue was dissolved in 60 mL Tris-HCl buffer (0.02 M, pH = 7.6) and the solution was

then gradually saturated with $(\text{NH}_4)_2\text{SO}_4$ at 0 °C to cause the fall-out of the proteins. Firstly, at 30 % $(\text{NH}_4)_2\text{SO}_4$ saturation level, undesired (contaminating) proteins were salted out and removed. Then, by further increasing $(\text{NH}_4)_2\text{SO}_4$ concentration and reaching 50 % saturation in the solution, a protein fraction with the highest CA enzyme activity was precipitated. This precipitated substance was centrifuged (12000 rpm, 10 minutes), dissolved in 40 mL Tris-HCl buffer (0.02 M, pH = 7.6) and subsequently dialysed to remove salts and other pollutants (e.g. ammonium sulfate residues). Dialysis has been done in diffusion dialysis bag (made of DEAE-cellulose). The sack containing the 40 mL enzyme solution was placed in a bucket filled with 10 L Tris-HCl buffer (0.02 M, pH = 7.6) (continuous stirring, room temperature). The conductivity in the dialysate was followed and the process was considered done once equilibrium was reached. Based on gravimetric analysis, the dialysed enzyme solution could be characterized with a 3.8 mg/mL dry matter concentration. Finally, the dialysed enzyme preparation was dried at 40 °C under vacuum by a Heidolph VV2000 Rotadest and stored in a refrigerator at 4 °C until use.

2.2. Characterization of the enzyme preparation

To determine CA activity, the modified Wilbur-Anderson method [13] was used. The measurements were validated by commercial CA enzyme (C3934) (Sigma-Aldrich, USA).

To test the activity of the enzyme preparation obtained by the process described in Section 2.1., 600 µL enzyme solution – well-defined amount of powdered CA enzyme preparation dissolved in 600 µL Tris-HCl buffer (0.02 M, pH = 8.3) – was added to 14.4 mL (0.02 M, pH = 8.3) Tris-HCl buffer. The mixture was thermostated at 4 °C and mixed vigorously (450 rpm). Thereafter, 6 mL substrate (distilled water saturated with CO_2) was

injected and the decrease of pH was recorded in the range of 8.2-7.2 as a function of time. Control tests without the enzyme were also carried out.

The activity (U) can be calculated from the times corresponding to 1 unit of pH decrease, as follows (Eq. 2):

$$U = \frac{t_0 - t_m}{t_m} \quad (2)$$

where, t_0 and t_m are the times in seconds measured for the control and the enzyme preparation, respectively.

From the activity (U) measured according to Eq. 2, the unit of $U \text{ mg}^{-1}$ was derived by taking into account the amount of enzyme preparation (mg dry mass) used during the activity measurement.

To confirm the presence of CA, SDS-PAGE was performed on a Cleaver Scientific Ltd, Nano-PAC – 300 gel apparatus with 4 % acrylamide stacking gel and 12.5% acrylamide running gel. The samples were treated with SDS and 2-mercaptoethanol before running. The proteins on the SDS-PAGE gels were stained with Coomassie Blue R-250 and visualised by a GelAnalyzer 2010a image analysis software.

2.3. SILM fabrication and gas permeation tests

Firstly, a 5.6 cm diameter circle was cut from hydrophobic Durapore® PVDF microfiltration membrane (Millipore Corporation, USA), placed in a Petri-dish and put in a vacuum desiccator for 1 h to remove the impurities (traces of water). In the meantime, the enzyme preparation (10 mg dried powder dissolved in 50 μL distilled water) was added to preliminary dried [4] 1950 μL [bmim][Tf₂N] ionic liquid (Sigma-Aldrich, USA). To help

dissolution and homogenization, vortexing and ultrasound sonication was applied in several steps. Then, the mixture was loaded by a syringe to the surface of PVDF membrane through a septum on the top of the desiccator, and carefully dispersed. To achieve the saturation of pores by the enzyme-water-IL solution, the vacuum inside the desiccator was allowed to grow up to ambient pressure conditions (the pressure increase aids the penetration of the solution into the pores). Gas permeation experiments were conducted in a device shown in Fig. 2, at a stable 40 ± 0.1 °C with single gases (CO_2 , H_2 , CH_4 and N_2), all of them having >99.9 vol% purity (Linde, Hungary).

In the beginning of each experimental run, the whole test rig (chambers, pipes) (Fig. 2) was flushed with the actual gas (supplied from cylinders) and the initial pressure in the feed chamber was set to 2 bar(a). At the same time, the permeate chamber contained the same gas at 1 bar(a) pressure. The permeation from the upstream- (high pressure) to the downstream (low pressure) compartment was followed by simultaneously measuring the pressure values in both sides. Data were registered in every 2 minutes until reaching equalized pressure conditions (loss of driving force).

The permeability values were calculated in accordance with the report of Neves et al. [10]. The theoretical selectivity ($S_{A/B}$) is a product of the permeability ratio of two different gases (A and B). Measurements – for performance comparison purposes – were carried out using SILM without the CA enzyme preparation (prepared only with IL and water).

In the course of the membrane stability tests, the SILMs were weekly tested with N_2 and subsequently with CO_2 . When the experiments with CO_2 were accomplished, the membrane was left in the permeation cell under equilibrated pressure conditions until the next week's inspection. The measurements were executed at least in duplicates and standard deviations were less than 5 %.

3. Results and Discussion

3.1. Results on CA enzyme preparation

As mentioned in Section 2.2., the reliability of modified Wilbur-Anderson method for measuring CA enzyme activity was checked. Accordingly, the activity of the commercial enzyme – known as 2500 U mg^{-1} – was determined as $2310 \pm 85 \text{ U mg}^{-1}$, which indicates fairly acceptable results.

The activity of the dried CA enzyme preparation from spinach was 5.8 U/mg , which, as a matter of fact, is considerably lower than that of its commercial counterpart. Nevertheless, it should be taken into account that spinach-derived CA in this work was obtained in a relatively simple way. Hence, although more purification steps in sequence were applied as described in Section 2.1., the CA enzyme obtained still probably contained impurities and might explain the differences.

To monitor the stability and storability of the dried enzyme preparation, its activity was regularly measured for several weeks under standardized conditions (Fig. 1). As it can be seen in Fig. 1, there was an initial loss of activity, but from the second week onwards, the successive values remained quasi-constant. Hence, because of this advantageous shelf life observed, it has been concluded that CA enzyme preparation is worthy to be applied in membrane gas separation experiments. This observation regarding the good storability of plant CA enzyme coincides well with the work of Pocker and Ng [14].

As for the structure of the CA enzyme from spinach leaves, it is reported that the enzyme (approx. 212 kDa molecular weight) consists of 8 subunits, including Zn ion on each [11]. The molecular weight of one subunit is ca. 26 kDa [11,15].

To verify CA content of our preparation from spinach, SDS-PAGE measurement was carried out, using the commercial carbonic anhydrase for comparison. As it is shown in Fig. 3, both in the case of CA standard (columns 10 and 11) and our samples (columns 2-9), there are significant bands at 26 kDa molecular weight, which is a positive feedback to affirm the presence of CA in the enzyme preparation derived from spinach. For the interested readers, more information about the structural features and other characteristics of spinach carbonic anhydrase can be found in the literature Ref. [16,17].

3.2. SILMs experiments combined with carbonic anhydrase enzyme preparation

SILMs were prepared with and without CA enzyme preparation content and systematically tested with pure CO₂, N₂, CH₄ and H₂ gases. In a biorefinery, organic matter can be converted under anaerobic circumstances into biomethane and/or biohydrogen. However, these fermentation end-products are obtained in a complex gaseous mixture, composing of CO₂ in notable amounts. Thus, for biofuel upgrading purposes, getting rid of carbon dioxide is required. Furthermore, CO₂/N₂ separation is a realistic issue of post combustion mixtures (flue gases), when oxygen is supplied from air.

These problems may be assisted by enzymatically-boosted SILMs, as discussed above in the Introduction section. After the separation, the selectively removed CO₂ may be utilized in different ways to restrict greenhouse gas emission to the environment. For instance, the reduced carbon footprint can be achieved by CO₂ sequestration to grow microalgae [18,19], to generate carboxylic acids [20,21] or to produce CH₄ via bio-electrosynthesis [22,23].

The permeability data obtained under the various conditions are presented in Fig. 4. As it can be drawn from Fig. 4, the gases applied are characterized by different permeabilities and moreover, it would appear that SILMs containing CA enzyme preparation ensured notably better performance in most cases. This indicates the significant contribution of the enzyme and shows also that the CA enzyme preparation from spinach was able to work successfully in the SILM system, even in this partly purified condition. Hence, it can be assumed that SILMs carrying CA enzyme only in smaller quantities could work well, and the required amount for the improvement of the separation can be provided by the enzyme preparation made of spinach biomass. This assumption concerning the need for only a small enzyme loading seems to be supported by the report of Neves et al. [10], where as low as 0.01 (m/m)% CA enzyme content led to a noticeable increment of SILMs separation characteristics in relation to CO₂ and N₂ gases. Therefore, the enzyme preparation made with simplified downstream processing in this study may have the potential to replace the higher cost commercialized CA. Nonetheless, it should be noted according to Suchdeo and Schultz [24] that the higher CA concentration in the membrane can be coupled with faster CO₂ conversion rates. This is consistent with the nature of enzymatic catalysis, where basically a direct relationship is established between rate of catalysed reaction and the dose of biocatalyst. However, the more enzyme is normally accompanied by an extra process cost.

In general, the gas transfer across SILMs is described by solution-diffusion theory [3]. Nevertheless, as a result of carbonic anhydrase addition, this regular mechanism is complemented by the specific affinity of the enzyme for CO₂ and a so-called facilitated transport is developed. This, because of the increment in partial driving force of this particular component, substantially improves the flux and the enhancement of selectivity feature can be realized. The phenomenon of chemical or biochemical facilitation is attributed

to the reversible reaction of carbon dioxide and the facilitating substance [25]. In the case of CA, it promotes the $\text{CO}_2\text{-H}_2\text{O}$ reaction by helping the formation of enzyme-bound Zn-OH- and bicarbonate generation [25].

In previous literature attempt with carbonic anhydrase and membranes for gas separation, Suchdeo and Schultz [24] reported that in membranes made with CA enzyme and NaHCO_3 , a more than 3-times higher CO_2 permeance was noticed compared to the enzyme-less system. Later, Bao and Trachtenberg [25] dedicated efforts to investigate various facilitated-transport supported liquid membranes (SLMs) for maximum CO_2 separation performance. It was found that among the few facilitator agents scoped, carbonic anhydrase together with alkaline carbonate yielded more attractive results under ambient conditions than diethanolamine did. Besides, Zhang et al. [26] prepared hollow-fiber membranes with hydrogel-immobilized carbonic anhydrase for CO_2 separation from gaseous mixtures. It has turned out that CA enzyme could keep 76 % of its activity during the experiments, proving the time-stability of the biocatalyst just like observed in this current work. More recently, a paper on enzymatic transport CO_2 -selective SILMs was communicated by Portuguese researchers [10]. It was deducted from the experiments that carbonic anhydrase – depending on the water activity of the solvent that CA was added to – was able to improve CO_2 solubility coefficient in the membrane by 20-30% that contributed to its selective transmembrane migration over other gas e.g. N_2 . This increased CO_2/N_2 theoretical selectivity as supported by the findings in this study too (Fig. 5).

It can be observed in Fig. 4 that the permeability of other gases besides CO_2 increased as well, although by various extents. The reason behind might be associated with the fact that the enzyme preparation was not completely pure, likely containing micro-pollutants (i.e. inorganic substances). This possibly caused micro-defects in the membrane structure and as a result, gas molecules (depending on their size) were able to pass through the membrane

relatively easier. From the permeability values the ideal selectivities were calculated (Fig. 5), where one can see that SILMs made with CA enzyme preparation from spinach possessed better features for CO₂/N₂ and CO₂/CH₄ gas pairs as compared to their conventional, enzyme-less counterparts. Meanwhile, the alteration of CO₂/H₂ selectivity was found to be insignificant.

In the last part of the measurements, the time-stability of the SILMs manufactured with enzyme system was addressed (Fig. 6). The outcomes of repeated permeability tests (covering 4 weeks) proved that the membrane integrity did not change over time. As a matter of fact, it can be drawn that the CA remained quite stable and re-usable for an extended period since CO₂ and N₂ permeabilities – as depicted in Fig. 6 – demonstrate only negligible changes, which can be explained by the experimental error.

It is important to point to the role of water content, or more importantly, to that of the water activity in the membranes, since it surely influences the efficiency of the CA enzyme [10]. It can be assumed that in applications with realistic gaseous effluents, the water content in the membrane is subject to change, depending on the properties of the feed. Thus, in a continuous gas separation process, the moisture of the inlet gas will affect the actual water contents. In addition, the water sorption/affinity characteristics of the specific IL used to fabricate the SILM will also determine water uptake and migration [27]. Consequently, in our future work, the impact of these factors will be examined to get a better comprehension about the behaviour of SILM prepared with CA enzyme.

4. Conclusions

Supported ionic liquid membranes combined with carbonic anhydrase enzyme were studied for selective CO₂ separation. CA enzyme preparation from spinach biomass was

successfully obtained and exhibited acceptable storability. In the course of batch permeation tests, it has turned out that the CA enzyme could enhance CO₂ transfer across the membrane, which, in most cases, has led to increased separation factors (CO₂/N₂: 30.28; CO₂/CH₄: 19.91) in comparison with membranes lacking this enzyme (CO₂/N₂: 23.84; CO₂/CH₄:15). Longer-term measurements (covering a 1 month period) indicated good membrane stability and imply that its properties should be further investigated in a continuous process.

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Figure captions

Fig. 1 – The stability of the enzyme preparation

Fig. 2 – Set-up of the membrane test equipment

1 – gas cylinder; 2,3,4,5 – valves; 6 – permeation cell; 7 – membrane; 8,9 – pressure transducers, 10,11 – data collection system

Fig. 3 – SDS-PAGE analysis of carbonic anhydrase preparation from spinach

Columns – 1,12: Protein molecular weight markers (20-120 kDa, 10-180 kDa, respectively); 2-9: samples of CA enzyme preparation from spinach (undiluted, 0.8x, 0.6x, 0.4x, 0.2x, 0.1x, 0.02x, 0.01x, respectively); 10,11: commercial (Sigma-Aldrich, USA) CA enzyme samples (4 mg/ml, 0.8 mg/ml, respectively). Sample loading was 10 μ l.

Fig. 4 – The permeability of gases in the SILMs

Columns: black – without CA enzyme preparation; grey – containing CA enzyme preparation

Fig. 5 – The theoretical selectivity values in the SILMs

Columns: black – without CA enzyme preparation; grey – containing CA enzyme preparation

Fig. 6 – Stability of the SILM + enzyme system

black squares – carbon dioxide; grey dots – nitrogen

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