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CO_2 hydration by immobilized carbonic anhydrase

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The enzyme carbonic anhydrase (isoform II) from bovine and human erythrocytes was immobilized using different covalent coupling methods on inert matrices. Immobilized carbonic anhydrase may enable concentration of CO_2 for Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase)-catalysed fixation in bioreactors. In the present study the activity of carbonic anhydrase with respect to hydration of CO_2 using soluble and immobilized enzymes was determined. The stability of the immobilization matrix, the properties of the immobilized enzymes subjected to a variation in operation variables and the activity profile with respect to storage are reported. Immobilization imparted greater thermal and storage stability and enhanced reusability.

Introduction

CA (carbonic anhydrase; EC 4.2.1.1), a zinc metalloenzyme, catalyses the reversible hydration of CO_2 and the dehydration of HCO_3^- and plays a significant role in processes such as pH homoeostasis, respiratory gas exchange, photosynthesis and ion transport [1–3]. It is widely distributed in the tissues of plants and animals [1,4–7], in several members of the Archea [8], in cyanobacteria [9,10] and in a variety of eubacteria [7,11]. The CA can be divided into three major groups based on the amino acid sequence: (i) the α group, which includes all CA from mammals and some from the algae and eubacteria; (ii) the β group, which includes CA enzymes from plants and some from algae, eubacteria and archaebacteria and (iii) the γ group, present in some archaebacteria, eubacteria and plants and which plays a role in acetate metabolism [12–14]. CA or CA-like activity plays an important role in photosynthesis and in the operation of the CCM (CO_2-concentrating mechanism) in achaebacteria, eubacteria and cyanobacteria [10]. Efficient photosynthetic inorganic carbon (C_i) assimilation by cyanobacteria, archaebacteria and some eubacteria at limiting available levels of C_i necessitates operation of CCM. As a process, CCM increases the CO_2 concentration around the primary carboxylating enzyme, Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) to levels several orders of magnitude above those present in the surrounding medium, enabling the enhancement of the rate of photosynthesis [1,10, 15]. The intracellular CO_2 concentration is elevated as a result of a two-step process, both steps involving CA activity. First, the light-dependent inorganic carbon-transport systems, which utilize both CO_2 and HCO_3^- activity, accumulate C_i in the cytosol. Second, the accumulated C_i, which is present mostly in the form of HCO_3^-, is dehydrated to CO_2, the substrate of Rubisco. The bicarbonate dehydration, catalysed by CA, occurs close to the active site of Rubisco [16].

A bioprocess enabling fixation of CO_2 in bioreactors employing recombinant Rubisco has been invented [17,18]. In this approach, modular devices are used, where in one module the CO_2 is fixed on RuBP (D-ribulose 1,5-bisphosphate) using Rubisco [18,19] and in the other module a cohort of enzymes regenerates RuBP from 3-phosphoglycerate. The bioprocess for fixation of Rubisco employs a highly enriched and relatively purified stream of CO_2 obtained after treatment of the emission stream. However, the ability to deliver concentrated CO_2 in aqueous soluble form at the active site of Rubisco still remains one of several major challenges. The mass-transfer rate of CO_2 from the gas phase to the aqueous phase is one of the rate-limiting steps. The residence time of CO_2 in the aqueous phase determines the fate of its being fixed by Rubisco in the biocatalytic fixation chamber. It is expected that a concentration step similar to that employed in microorganisms can be used in bioreactors employing immobilized CA. CA is known to play a critical role in CCM in cyanobacteria, archaebacteria and photosynthetic eubacteria. Although a few cases of immobilization of CA have been reported [20–22], they are not intended for use in bioengineered systems for CCM or for similar applications.

Key words: carbonic anhydrase, CO_2 concentration, CO_2-fixation bioprocess, immobilized carbonic anhydrase, immobilized carbonic anhydrase bioreactor.

Abbreviations used: CA, carbonic anhydrase; CCM, CO_2-concentrating mechanism; C_i, inorganic carbon; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, D-ribulose 1,5-bisphosphate; WA unit, Wilbur–Anderson unit; DCC, dicarboxycarbodi-imide.

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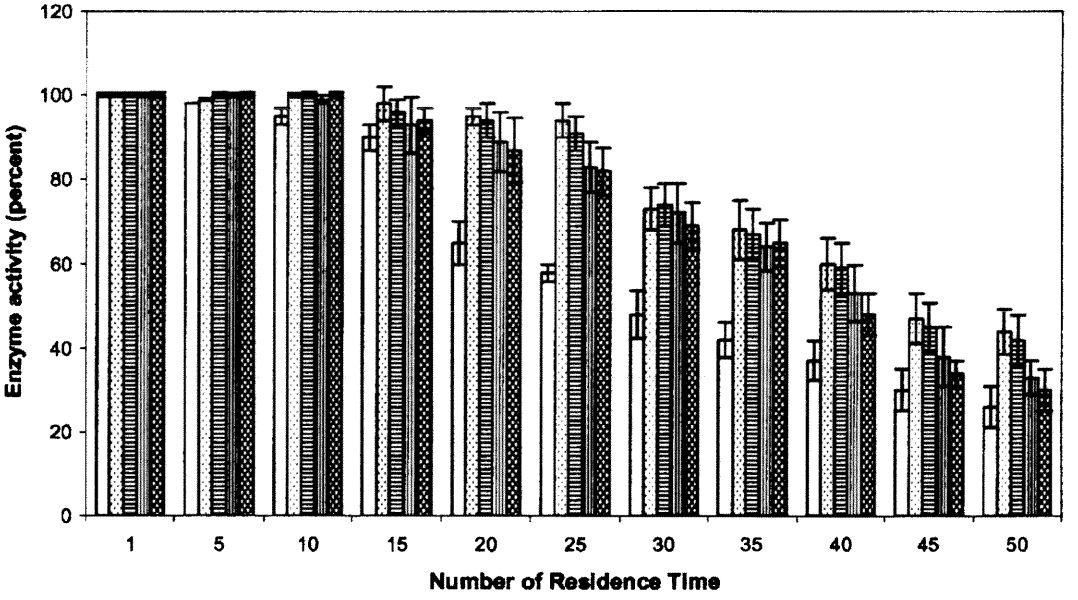

Figure 1 Determination of the loss of activity with respect to the number of use cycles for soluble and immobilized enzymes

Activity was measured as described in the Materials and methods section. Empty bars, soluble enzyme. Immobilized enzymes: bars with dots, DCC-coupled, bars with horizontal stripes, carboxyl-coupled; bars with vertical stripes, CNBr-coupled; bars with checks, methacrylate-co-polymer-immobilized. Three independent experiments were performed (means ± S.D. are shown).

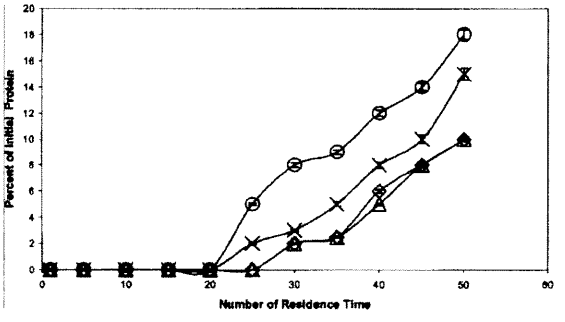

Figure 2 Determination of the amount of leached protein as a function of residence-times for CA II immobilized by different methods

The total initial protein load was taken as 100 % and amounts of leached protein determined using the Bradford method. Data are expressed as the percentage of initial protein. Bovine CA II: ◇, DCC-coupled; △, carboxy-coupled; ×, CNBr-coupled; ○, methacrylate-co-polymer-immobilized enzyme.

were nearly identical (Figure 2). The leaching is presented as a percentage of the total initial coupled protein; however, comparison of leached protein (Figure 2) with the percentage of activity lost (Figure 1) does not enable us to account for the total loss of activity due to leaching. This is also true for human erythrocyte CA II (results not shown), the loss of enzyme activity in prolonged use is probably due to the modification of enzymic protein resulting in a loss of catalytic ability. The exact reason for the leaching of covalently coupled enzymes out of the matrix, however minute the amount, remains to be understood. Leaching increased with an increase in residence-cycle time and became significant beyond cycles 20–25 (Figure 2). The rate of leaching was increased with a further increase in residence-time cycle but it accounted for only about 10% (for DCC- and carboxy-coupled enzymes) and a maximum of about 18% (for methacrylate-co-polymer-coupled enzyme) of the original load value of the enzyme (Figure 2).

The pH profile of immobilized CA

The activity of bovine CA II as a function of pH was determined, which has been presented in Figure 3. The pH optimum for soluble CA II was between 8.5 and 9.0, whereas that of the immobilized enzymes showed a shift of about 1 pH unit; that is, pH 9.5–10.0. This shift in pH optimum is perhaps due to the change in microenvironment provided by the immobilization. The pH–activity profiles of enzymes near the optima were similar for proteins immobilized by all four methods. The decrease in activity on either side of the plateau was sharp for soluble enzyme compared with immobilized enzymes. The immobilized enzyme pH–activity profile showed a shift to the right compared with the soluble enzyme (Figure 3).

Temperature-activity profile

The residual activity as a function of time was determined at different temperatures. Figure 4 presents temperature–activity profiles of the soluble and immobilized enzymes.

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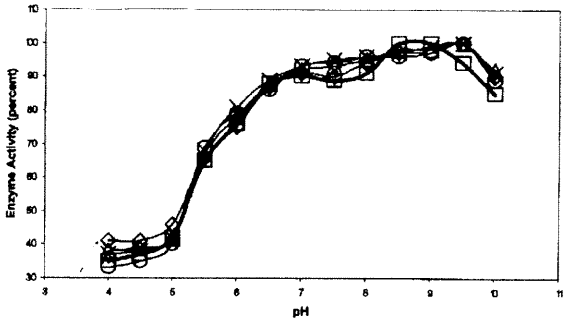

Figure 3 pH–activity profiles of soluble and immobilized CA II

The enzymes were subjected to a buffer of pH 7.0–10.0 for activity determination. □, Soluble enzyme; ◇, DCC-coupled; △, carboxy-coupled; ×, CNBr-coupled; ○, methacrylate-co-polymer-immobilized enzyme.

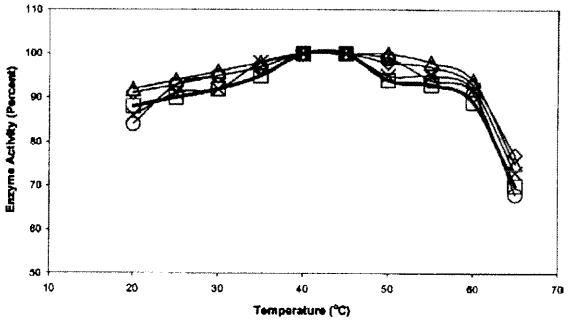

Figure 4 Temperature–activity profiles of soluble and immobilized bovine CA II

The enzymes were subjected to a temperature range of 20–65 °C as mentioned in the Materials and methods section and activity was determined. □, Soluble enzyme; ◇, DCC-coupled; △, carboxy-coupled; ×, CNBr-coupled; ○, methacrylate-co-polymer-immobilized enzyme.

To determine the effect of temperature on the rate of deactivation of the enzyme we tested 20 and 65 °C, the lowest and highest temperatures at which bovine CA II activity has been determined in the present investigation.

The natural logarithm (ln) of the ratio of residual activity (%) at a specified temperature to initial activity (taken as 100%) was plotted against the time of incubation. The resulting plots are presented in Figure 5. The rates of deactivation for soluble and immobilized enzymes were extremely low at 20 °C, shown by the linear and almost identical slopes. However, at 65 °C the rate of deactivation was significantly higher for both soluble and immobilized enzymes. Deactivation of the soluble enzyme at 65 °C was fastest than that of the immobilized enzymes. The DCC- and carboxy-coupled enzymes had the lowest rates of deactivation (Figure 5), which is consistent with their temperature-stability profiles (Figure 4). The ratios of the specific rate of deactivation at 65 °C for soluble enzyme to DCC- and carboxy-coupled bovine CA II were 1.89 and 1.75 (the ratio of the slopes of the rate of deactivation curves), while the ratios of the

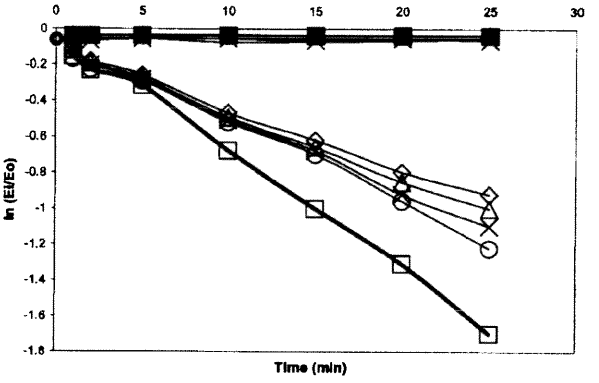

Figure 5 Deactivation kinetics of soluble and immobilized bovine CA II

For determination of deactivation, the pre-incubated enzyme and assay mixture at the specified temperature (20 and 65 °C) was added and incubated for indicated time intervals and activity was determined as described in the Materials and methods section. The natural logarithm (ln) of the ratio of residual activity at the specified temperature at the time of determination (Et) to that of the original activity (Eo; taken as 100%) was plotted against time. □, ■, Soluble enzyme; ◇, ◆, DCC-coupled; △, ▲, carboxy-coupled; ×, ✖, CNBr-coupled; ○, ●, methacrylate-co-polymer-immobilized enzyme at 65 and 20 °C respectively.

slope of soluble enzyme to that of CNBr- or methacrylate-co-polymer-immobilized enzymes were 1.6 and 1.48 respectively. Thus specific rate of deactivation for soluble enzyme was the most pronounced followed by the methacrylate-co-polymer-, CNBr-, carboxy- and DCC-immobilized enzymes (Figure 5). These findings are also consistent with the temperature–stability profiles of the respective immobilized enzymes (Figure 4). The thermal stabilities of the DCC- and carboxy-coupled enzymes were therefore superior compared with CNBr-coupled, methacrylate-co-polymer-immobilized or soluble enzymes.

Storage

The soluble and immobilized bovine CA IIs was stored at 20 and 35 °C. The activity retention of immobilized CA II was higher than that of soluble enzyme at corresponding temperatures. The DCC- and carboxy-coupled immobilized enzymes showed greater activity retention on storage at 35 °C than the CNBr-activated, methacrylate-coupled or soluble enzymes (Figure 6).

Discussion

Anthropogenic emission of CO_2 into the atmosphere has been implicated in many unintended environmental consequences [41,42]. The capture of CO_2 once emitted and diffused in the atmosphere is a difficult process, both labour- and cost-intensive. Capture of CO_2 at the emission point and the abatement of CO_2 pollution at the emission source is

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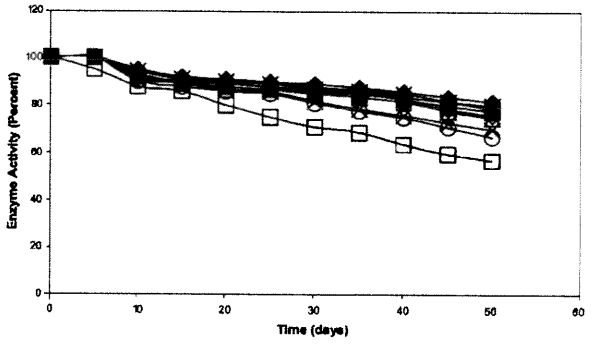

Figure 6 Storage stability of soluble and immobilized CA II

The enzymes were stored at 20 and 35 °C and activity determined after the indicated time intervals. Residual activity was determined at 25 °C. □, ■, Soluble enzyme; ◇, ◆, DCC-coupled; △, ▲, carboxy-coupled; ×, ✖, CNBr-coupled; ○, ●, methacrylate-co-polymer-immobilized enzyme at 35 and 20 °C respectively.

therefore highly desirable and has great potential for minimizing the cost of capture. It has been established that Rubisco in the solution phase could be used to fix CO_2 [17–19,23]. The continuous fixation of CO_2 would necessitate regeneration of RuBP. Continuous RuBP-regeneration processes have been improvised [17,18] and are being developed which would facilitate continuous fixation. However, for the continuous fixation of CO_2 at stationary emission sources using Rubisco would necessitate the presentation of CO_2 near the enzyme's active site. Rubisco is a relatively slow-acting enzyme and in cyanobacteria, archaebacteria and photosynthetic eubacteria an effective CCM exists that employs an efficient capture step using CA, one of the fastest-acting enzymes known. Such a concentration mechanism has the potential to be used for the bioengineered CO_2-fixation process. In order to achieve efficient CO_2 capture, a coupled system of immobilized CA with immobilized Rubisco [19] would be required. The relative mechanical stability of the immobilization matrix in the previously reported immobilized CA approaches did not allow withstanding necessary physicochemical changes.

Previous experimental studies on the thermal stability of soluble erythrocyte CA of the enzyme showed that the enzyme was found to be very stable below 60 °C; however, between 60 and 65 °C a drastic decrease in biological activity was observed [43]. This was reproduced in our investigation as well. However, the immobilization did not significantly alter the temperature optimum (Figure 4). Studies have been reported on a cellulose nitrate-encapsulated, silicone-rubber-membrane-immobilized form of CA II, evaluating thermal, operational and storage stability. The extent of enzyme activity in free solution and in the encapsulated form was determined after long-term storage at 4, 37 and 50 °C. The thermal-stability tests showed a significantly greater degree of retained enzyme activity in the encapsulated form over the free enzyme in solution, at all temperatures but especially at higher temperatures and when the enzyme was stored for extended periods. In the operational stability tests, the CO_2-removal efficiency of the treated membrane was not degraded, and stayed significantly higher than the untreated membrane for extended time periods. These studies, besides demonstrating improved thermal stability, illustrate that the immobilized CA retained improved CO_2-removal efficiency compared with the soluble counterpart for extended periods [20]. The mechanical and thermal stabilities of DCC- and carboxy-coupled CA presented here appear to be superior to the other methods of immobilization. These enzymes are expected to be suitable for application in bioreactors for CO_2 concentration. Our efforts towards utilizing the immobilized CA in applied bioprocesses are in progress.

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