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Technical Note

Purification of carbonic anhydrase from bovine erythrocytes and its application in the enzymic capture of carbon dioxide

Joana da Costa Ores, Luisa Sala, Guido Picaluga Cerveira, Susana Juliano Kalil *

Universidade Federal do Rio Grande, Escola de Química e Alimentos, Rua Eng. Alfredo Huch, No. 475, P.O. Box 474, 96201-900 Rio Grande – RS, Brazil

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ABSTRACT

This work presents a study of industrially applicable techniques to obtain a biologically supported carbon dioxide capture system, based on the extraction of carbonic anhydrase from bovine blood. Carbonic anhydrase is a metalloenzyme which catalyzes the reversible hydration of carbon dioxide. The objective of this study was to establish conditions to obtain carbonic anhydrase from bovine erythrocytes and apply it in the capture of carbon dioxide. To achieve this, two different purification techniques were evaluated: one by extraction with the organic solvents chloroform and ethanol, where different solvent proportions were studied; and the other by ammonium sulfate precipitation, testing percent saturations between 10% and 80%. Carbon dioxide was enzymatically captured by its precipitation as calcium carbonate with the enzyme obtained by both techniques. The enzyme extracted by ethanol and chloroform showed an activity of 2623 U mL^{-1}, recovery of 98% and purification factor of 104-fold. That precipitated by ammonium sulfate showed an activity of 2162 U mL^{-1}, recovery of 66% and purification factor of 1.4-fold using 60% ammonium sulfate saturation. The results obtained in the carbon dioxide capture experiments showed that the carbonic anhydrase extracted in this study not only enhanced the hydration of CO_2, but also promoted the formation of $CaCO_3$.

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1. Introduction

Every year the meat sector consistently produces large quantities of waste including offal, bones, blood, skin, horns and hoofs. This waste represents a cost for the meat processing sector as well as being an environmental pollution problem (Bernardini et al., 2011). The animal blood produced in slaughterhouses represents the most problematic by-product of the meat industry, due to the large volumes generated and its very high pollutant load. Regulations restrict the direct disposal of blood waste to wastewater treatment plants, so different elimination techniques (drying, incineration, etc.) are usually used to treat this by-product (Hoyo et al., 2008).

The blood is one of the most important by-products produced in the slaughter of animals, and has high biological value (Viana et al., 2005). Bovine blood and its fractions (plasma and erythrocyte) represent a potential source of large amounts of edible nutritional proteins and essential amino acids (Márquez et al., 2005). Meat wastes from slaughtering and processing are a rich source of proteins and may therefore be viewed as a potential starting material for the production of high value-added products (Bernardini et al., 2011), such as the enzyme carbonic anhydrase.

Carbonic anhydrase (EC 4.2.1.1) is a zinc-containing metalloenzyme that is widely distributed in nature and catalyzes the reversible hydration of CO_2 to HCO_3^- with great efficiency: $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$ (Ozensoy et al., 2004; Nishita et al., 2005, 2007; Dogan, 2006; Wei et al., 2006). So far, sixteen isozymes have been described that differ in their subcellular localization, catalytic activity and susceptibility to different classes of inhibitors (Bayram et al., 2008).

Carbonic anhydrases (CA) are produced by a variety of tissues where they participate in a series of important biological processes such as acid–base balance, respiration, carbon dioxide and ion transport, bone reabsorption, ureagenesis, gluconeogenesis, lipogenesis, body fluid generation (Bayram et al., 2008), photosynthesis, calcification (Smith and Ferry, 2000) and the production of aqueous humor (Centofanti et al., 1997).

While CO_2 is not in itself a particularly strong greenhouse gas (compared to, for example, methane), the contribution to the atmosphere since the beginning of the industrial revolution through the burning of fossil fuels has been very considerable. Thus, CO_2 capture and sequestration has been identified as the critical enabling technology to reduce CO_2 emissions significantly (Dilmore et al., 2009; Ramanan et al., 2009).

Biologically based capture systems are a potential route for improvement in CO_2 capture technology. These systems are based on the naturally occurring reactions of CO_2 in living organisms, and one of these possibilities is the use of enzymes, such as CA

* Corresponding author. Tel.: +55 53 32338754.
E-mail addresses: Kalildqmsjk@furg.br, dqmsjk@furg.br (S.J. Kalil).

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(Figueroa et al., 2008). CA has potential for CO_2 capture, presenting itself as an alternative industrial process, for reducing CO_2 emissions to the atmosphere. The capture can be done via CO_2 hydration by the enzyme and subsequent chemical fixation in the form of carbonate minerals, which have the advantage of being naturally present in nature (Mirjafari et al., 2007).

The few studies using CA for CO_2 capture used highly purified enzyme preparations or sources difficult to scale up, such as human blood (Bond et al., 2001; Mirjafari et al., 2007; Dilmore et al., 2009; Davy, 2009; Favre et al., 2009), which increase the cost of the process. The feasibility of the capture process resides initially in obtaining the enzyme using only a few steps, but resulting in an efficient enzyme for dioxide carbon capture from a viable source, such as bovine blood.

The main source of interference in the purification of CA from erythrocytes is the hemoglobin. One well-known method for removing this is by its denaturation with chloroform and ethanol. However, studies that use this method do not aim at using the enzyme in CO_2 capture systems (Meldrum and Roughton, 1933; Armstrong et al., 1966; Tanis and Tashian, 1971; Nishita et al., 2005).

Current research usually employs the hemolysate directly on an affinity chromatography column (Sinan et al., 2007; Bayram et al., 2008; Alp et al., 2010), since this provides the high degree of purity needed for characterization and/or inhibition studies, eliminating the use of sequences of different techniques. However, for industrial applications high degrees of purity are not required.

Thus it is necessary to study the capture with economically feasible enzyme extracts with lower degrees of purity, which can be obtained by industrially applicable purification techniques, thus reducing the costs of capturing and sequestering of CO_2.

This study provides a potential biologically based capture system, which uses an original slaughterhouse by-product – blood, to provide a possible alternative solution to mitigate the global climate changes caused by greenhouse gases. Further to the environmental issue, this process adds value to the most problematic by-product of the meat industry. In the present research, the use of industrially applicable techniques for the purification of the CA extracted from bovine erythrocytes, and its application in the enzymatic capture of carbon dioxide were studied. The enzyme was partially purified in two different ways: extraction with ethanol and chloroform and ammonium sulfate precipitation. The enzyme obtained by both techniques was used in the capture of carbon dioxide in the form of calcium carbonate. To our knowledge, this was the first time a partially purified enzyme obtained in just a few steps, was used in the capture of carbon dioxide.

2. Materials and methods

2.1. Obtaining the blood samples

The bovine blood samples were obtained from slaughtered animals at a slaughterhouse in the region of Pelotas/RS, Brazil. These samples were placed in sterile bottles with the anticoagulant citrate phosphate dextrose adenine in a 1:7 ratio (anticoagulant:blood). The samples were kept under refrigeration during transport and stored at 10 °C until analyzed.

2.2. Enzyme extraction

The samples were centrifuged at 4700 g for 10 min at 4 °C, and the white blood cells and plasma removed. The compacted red cells (erythrocytes) were washed three times with saline (0.9% NaCl) and then lysed with deionized water at 4 °C followed by vigorous agitation. The plasma membrane was removed by filtration.

2.3. Purification of carbonic anhydrase by extraction with chloroform and ethanol

As shown in Table 1, four trials with different proportions of chloroform and ethanol were evaluated for the extraction and purification of the enzyme. The suspensions were stirred and centrifuged to remove excess chloroform and precipitate, and the supernatant filtered to remove any remaining precipitate. The purification factor (PF), percent recovery (%R) and hemoglobin concentration were evaluated in triplicate.

2.4. Carbonic anhydrase purification by ammonium sulfate precipitation

The CA enzyme extract was placed in conical flasks, and different amounts of ammonium sulfate gradually added to obtain saturations between 10 and 80%. After resting for 14 h at 10 °C, the samples were centrifuged at 4700 g and 4 °C for 30 min, the supernatant separated and the precipitate re-suspended in deionized water. The enzymatic activity and total protein content were determined in both the supernatant and the precipitate. In the trials showing a precipitate, the PF, %R and hemoglobin concentration were evaluated in triplicate.

2.5. Carbon dioxide enzymatic capture

The CA purified by both techniques was used as a catalyst in the capture of carbon dioxide by precipitation in the form of $CaCO_3$. The reaction mixture consisted: 7.5 mL of the purified enzymatic extract, 7.5 mL of 1.2 M tris-HCl buffer pH 10.5 solution containing 4.5% (w/v) $CaCl_2$ and 30 mL of the CO_2 solution. The CO_2 solution was prepared by bubbling deionized water with gaseous CO_2 at a pressure of ~0.1 MPa and temperature of 5 °C. The reaction started when the CO_2 solution was added into the flask that was immediately closed with a sealing film. In all experiments the temperature was maintained at 5 °C. The mixture was then filtered and dried after 8 and 120 min of reaction to determine the weight of $CaCO_3$ precipitated (enzymatic assay). At the same time samples were prepared by replacing the enzyme with deionized water (non-enzymatic assay) and the results expressed as the difference between the values obtained in the enzymatic assay and those obtained in the non-enzymatic assay (Δ_{CaCO3}), calculated according to Eq. (1). For use in CO_2 capture, the enzyme purified by ammonium sulfate precipitation was dialyzed to remove the salt. The reaction was performed at 4 °C.

$$\Delta_{CaCO_3} = m\ CaCO_3\ EA - m\ CaCO_3\ NEA \quad (1)$$

where m $CaCO_3$ EA is the weight of $CaCO_3$ in the enzymatic assay (g) and m $CaCO_3$ NEA is the weight of $CaCO_3$ in the non-enzymatic assay (g).

2.6. Analysis

The activity of the CA was measured from the hydration of carbon dioxide (CO_2) as described by Wilbur and Anderson (1948),

Table 1
Proportions of water, ethanol and chloroform used in the methods studied.

Trial	Solvent proportions	References
1	1.1 Water:0.53 ethanol 95%:0.65 chloroform	Armstrong et al. (1966)
2	0.6 Water:0.4 ethanol:0.5 chloroform	Meldrum and Roughton (1933)
3	0.8 Ethanol 40%:0.4 chloroform	Roughton and Booth (1946)
4	3 Water:0.5 ethanol 95%:0.62 chloroform	Tashian et al. (1972)

with a slight modification. The reaction mixture consisted of 20 mM tris buffer pH 8.3, the enzyme and CO_2 solution. The CO_2-hydratase activity was recorded as Wilbur–Anderson enzymatic activity units (U), defined as $[(T_0/T)] - 1$, where T_0 and T are the times for a pH change to occur in the non-enzymatic (T_0) and enzymatic (T), reactions, respectively. The total hemoglobin concentration was estimated spectrophotometrically from the absorbance at 546 nm (Suh et al., 2005). The total protein concentration was determined by the method of Lowry et al. (1951), using bovine serum albumin as the standard protein.

2.7. Determination of the recovery and purification factor

The purification efficiency of the CA enzyme was assessed from the %R (Eq. (2)) and PF (Eq. (3)), which considers the increase in specific activity after the purification step.

$$\%R = \left[\frac{\Sigma(A_P V_P)}{(A_E V_E)}\right]100 \quad (2)$$

$$PF = \left[\frac{\Sigma(A_P V_P)/\Sigma(P_P V_P)}{(A_E V_E)/(P_E V_E)}\right] \quad (3)$$

where A_P (U mL^{-1}), V_P (mL) and P_P (mg mL^{-1}) are the enzyme activity, volume and protein concentration after the purification step, respectively; and A_E (U mL^{-1}), V_E (mL) and P_E (mg mL^{-1}) are the enzyme activity, volume and protein concentration of the crude extract, respectively.

2.8. Statistical analysis

The results were analyzed by the analysis of variance followed by the Tukey test, considering a 95% confidence level ($p < 0.05$).

3. Results and discussion

3.1. Purification of carbonic anhydrase by extraction with chloroform and ethanol

Four trials were evaluated for the extraction and purification of the enzyme by hemoglobin precipitation with ethanol and chloroform. The results are presented in Table 2, where the same letters indicate no significant difference between the means at the 95% confidence level.

The values for hemoglobin were not presented in Table 2, because almost 100% of the hemoglobin was removed in all the trials, and only traces were detected. Meldrum and Roughton (1933) also detected only traces of hemoglobin after extraction with ethanol/chloroform when separating hemoglobin from the extract of bovine erythrocytes.

Table 2 shows that the PF was statistically similar ($p < 0.05$) for trials 1, 2 and 3. With respect to %R, the highest value for this response (98%) was obtained in trial 2, which was statistically different from the others, as also the enzymatic activity (2623 U mL^{-1}).

Table 2
Means and standard deviation obtained for enzyme activity, recovery and purification factor in the purification of carbonic anhydrase by extraction with ethanol and chloroform.

Trial	A (U mL^{-1})	P (mg mL^{-1})	Recovery (%)	Purification factor
1	793 ± 205^c	0.7 ± 0.03^b	34 ± 7^c	$96 \pm 22^{a,b}$
2	2623 ± 311^a	1.5 ± 0.04^a	98 ± 11^a	104 ± 10^a
3	1845 ± 213^b	1.4 ± 0.02^a	75 ± 5^b	106 ± 12^a
4	497 ± 164^c	0.6 ± 0.03^b	43 ± 17^c	61 ± 17^b

A – activity, P – protein. Same letters indicate no significant difference between the means ($p < 0.05$).

Note also that the highest PF (106-fold) in the third trial, was statistically similar to that obtained in trial 2.

Meldrum and Roughton (1933) obtained %R values of about 80% when extracting CA from horse erythrocytes, a result lower than that obtained in the present work, which was 98%. Pol et al. (1990) attempted to purify human erythrocyte CA, performing a sequence of purification techniques: three-phase partition, ion exchange chromatography, gel permeation and affinity chromatography. The partition was carried out by adding *t*-butanol and chloroform, followed by centrifugation, filtration, addition of ammonium sulfate and dialysis. The authors achieved a %R of 96% and PF of 68-fold using this procedure. These values were lower than those obtained in the present work, which were 98% and 104-fold, respectively.

3.2. Carbonic anhydrase purification by ammonium sulfate precipitation

When using the ammonium sulfate precipitation technique, one must know the ranges of salt concentration where the protein of interest and any interferents precipitate, thus allowing for purification of the biomolecule. In the present case, the tests were carried out by varying the ammonium sulfate saturation between 10% and 80%.

There was no protein precipitation at percent saturations between 10% and 50%, and therefore the activity was not analyzed at these percentages. At percent saturations between 70% and 80%, it was observed that both the enzyme (CA) and the hemoglobin precipitated completely, and thus it was not possible to separate one from the other. However, in the test using 60% ammonium sulfate saturation, much of the CA precipitated, but a considerable amount of the enzyme remained in the supernatant.

Based on these results, new tests were carried out in triplicate with 60% and 65% ammonium sulfate saturation, and the means and standard deviation obtained for the enzyme activity, PF and %R are presented in Table 3. The same letters indicate no significant difference between the means at a 95% confidence level. The percent hemoglobin recovered was also monitored, since this represents the major contaminant, being present in high concentrations in the enzyme extract, which makes it difficult to separate from the CA.

It can be seen from Table 3 that the values for %R and PF in the supernatants obtained for 60% and 65% saturation, were statistically similar ($p < 0.05$), as also the %R obtained in both precipitates. Moreover, the values obtained for the hemoglobin recovered in the supernatant and in the precipitate (60% saturation) were statistically equal. A saturation of 60% was the one that gave the best results for the PF (1.4-fold) and %R (66%). A saturation of 65% was not satisfactory for enzyme purification, because almost all the hemoglobin was precipitated along with the CA, not being possible to separate the two molecules. The PF values achieved using this process were lower than those obtained by the ethanol/chloroform method presented first. However, depending on the intended application, high degrees of purification are not needed, but it is necessary to verify the influence of any residual hemoglobin on the performance of CA for CO_2 capture purposes.

3.3. Enzymatic capture of carbon dioxide

CA catalyzes the hydration of CO_2, and consequently hydrogen ions are transferred between the active site of the enzyme and the surrounding buffer. This results in a change in pH. Therefore measuring the pH is a viable method to monitor the progress of this enzymatic reaction.

Fig. 1 shows the decrease in pH during the assay for CA using the enzyme obtained by ethanol/chloroform extraction (using the

Table 3
Enzymatic activity, purification factor and recovery of the carbonic anhydrase precipitated with ammonium sulfate and the recovery of hemoglobin.

	Volume (mL)	A (U mL^{-1})	P (mg mL^{-1})	Hb (%)	R (%)	FP
Extract	10	2227	118	–	100	1
60%						
Supernatant	9.5	107 ± 18[c]	59	38 ± 3[b]	4.5 ± 0.8[b]	0.1 ± 0.01[b]
Precipitate	6.8	2162 ± 205[a]	84	43 ± 3[b]	66 ± 6[a]	1.4 ± 0.2[a]
65%						
Supernatant	5.4	19 ± 6[c]	8	2.4 ± 0.4[c]	0.5 ± 0.1[b]	0.1 ± 0.03[b]
Precipitate	10.7	1600 ± 258[b]	88	78 ± 0.03[a]	77 ± 14[a]	1.0 ± 0.1[c]

A – activity, P – protein, Hb – recovery hemoglobin, R – brecovery, FP – purification factor. Same letters indicate no significant difference between the means ($p < 0.05$).

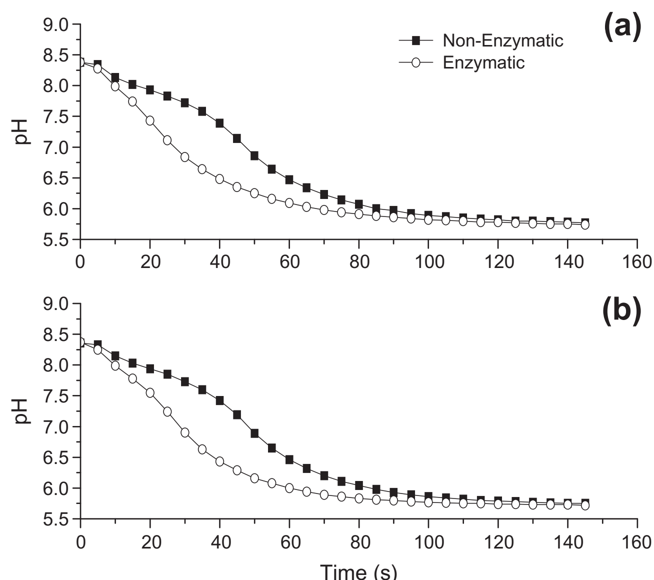

Fig. 1. Enzymatic hydration of CO_2 using the (a) ethanol/chloroform extract and (b) dialyzed precipitated extract.

Table 4
Results obtained in the enzymatic precipitation of carbon dioxide.

Purification technique	Time (min)	Δ_{CaCO_3} (g)	Standard deviation
Ethanol/chloroform extraction	8	0.0685[a]	0.0078
	120	0.0776[a]	0.0057
Sulfate precipitation and dialysis	8	0.0613[a]	0.0053
	120	0.0717[a]	0.0061

Same letters indicate no significant difference between the means ($p < 0.05$).

proportions from trial 2 – Section 3.1), and by precipitation with ammonium sulfate (using 60% saturation). The extracts were diluted 1:200 in both cases. The curves show that this enzyme was a very effective catalyst for hydration of CO_2.

As shown in Table 4, where same letters indicate no significant difference between the means at a 95% confidence level, the CA obtained by both techniques enhanced the formation of $CaCO_3$. The amounts of calcium carbonate precipitated after 8 and 120 min were statistically equal and there was no difference in the total mass of $CaCO_3$ precipitated by the two purified extracts. This behavior was also observed by Mirjafari et al. (2007) and Favre et al. (2009). The authors demonstrated that the total mass of $CaCO_{3(s)}$ precipitated do not depend on the concentration of the enzyme which, as a catalyst, can only change the kinetics to reach equilibrium that that occurs in the first minutes of reaction, not the equilibrium thermodynamics.

The present results are superior to those achieved by Mirjafari et al. (2007), who carried out the enzymatic precipitation of carbon dioxide into calcium carbonate using a highly purified enzyme. The authors obtained 0.02 g of calcium carbonate using a 6 μM enzyme solution, as against 0.06–0.07 g obtained in the present work, with the additional advantage of using a partially purified enzyme, which could have been a limiting factor in the feasibility of the process.

4. Conclusions

The purification of CA by ethanol/chloroform extraction resulted in values of 2623 U mL^{-1} for enzyme activity, a %R of 98% and a PF of 104-fold, using a ratio of 0.6 mL of deionized water, 0.4 mL of ethanol and 0.5 mL of chloroform per mL of erythrocytes.

In the purification of CA by ammonium sulfate precipitation, an activity of 2162 U mL^{-1}, PF of 1.4-fold and %R of 66% were obtained using 60% saturation.

The purified enzyme extracts obtained by both extraction procedures showed the potential for use in carbon dioxide capturing processes. Therefore, these purification techniques represent feasible alternatives for the industrial capture of CO_2.

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