Inclusion of partly purified protease from *Abrus precatorius* Linn in Ca-alginate gel beads

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**ABSTRACT**

*Abrus precatorius* is an edible endemic plant in Cameroon. In the northern regions of Cameroon, the leaves of this plant are used as traditional medications, for the liquefaction of wort obtained after mashing of sorghum or millets for processing the local fermented beer called bili-bili, and to sweeten gruels made from red millet. *Abrus precatorius* has also been explored as a potential source of proteolytic enzymes. In this work, a partially purified protease from the leaves and stems of the plant was immobilized in a calcium alginate gel beads and the protease activity (PA), investigated using a central composite design plan under the conditions: alginate content (1-5%) (w/v), enzyme/alginate ratio (10-30%) (v/v), and CaCl2 concentration (100–400 Mmol/L). Results showed that the optimum activity was obtained for an alginate content of 1% (p/v), an enzyme/alginate ratio of 10% (v/v) and a CaCl2 concentration of 400 Mmol/L. At the optimum, kinetic parameters of the immobilized enzyme (KM: 6.83 mg mL⁻¹; Vmax: 46.95 g.L⁻¹.min⁻¹) were comparable to those of free enzyme (KM: 1.26 mg mL⁻¹; Vmax: 153.85 g.L⁻¹.min⁻¹). The beads have been preserved at room temperature and reused for four days before losing 88% of its activity. This indicates that the protease activity of the leaves and stems of *A. precatorius* can be immobilized in calcium alginate beads and preserve four days at ambient temperature.

**1. Introduction**

Enzymes are protein in-nature catalysts that help reducing activation energy of reactions, without changing the thermodynamic equilibrium constant of a chemical transformation. They induce kinetic rate accelerations, often exceeding factors of $10^{12}$ relative to the rate of the uncatalyzed reaction [1]. They can engage many different chemical transformations, can be produced on a large scale, operate at ambient temperatures and close to neutral pH. These properties interest generations of scientists and engineers over the past century and facilitated their use as industrial catalysts [2]. Industrial enzymes can be extracted from natural sources (animal, plant, micro-organisms), more often through large-scale microbial fermentation processes. Enzymes from plant such as actinidin, α-amylase, β-amylase, β-glucanase, ficin, lipoxygenase, papain have been used in industrial processes for a long time in biotechnology (detergent, dairy, leather, baking, brewing, fuel ethanol, starch, textiles industries) and pharmacology as part of the final product or used as processing aids by manufacturers [1]. Over 50% of the sales are of proteolytic enzymes. In Cameroon, the importation of certain industrial proteases is expensive. Their transportation and production from animal sources are quasi-impossible due to the legislation which bans the slaughter of calves under 4 years old [3]. As an alternative source of proteases to clot milk, soften meat or produce beverages, populations use extracts from local plants as barks of trees (*Acacia albida*), sap (*Calotropis procera*), fruits (*Adenolichos anchaitae*) [7], the leaves and stems (*Abrus precatorius*) [4, 5].

*A. precatorius* is a legume commonly known as *danrai* in the Adamawa Region of Cameroon [5]. Proteases from leaves and stems of this plant have already been extracted and be partially purified. These extracts have proved to be very active on various substrates (*Ricinodendron houdeletii* defatted flour, gelatin, bovine serum albumin, casein, hemoglobin) [6]. The partially purified enzyme was not stable for a long time. It could take less than a week to lose it’s entire activity, though the conditions of preservation of their activity have not been fully elucidated.

The ability of an enzyme to convert substrate into a product is referred to as enzyme activity. The activity of an enzyme is a function of the intrinsic nature of the enzyme itself, as well as environmental factors such as substrate concentration, temperature, and pH [1]. Many methods of preservation such as freezing, lyophilization, crystallization, precipitation and immobilization have been developed [2]. Immobilization...
enhance the stability of an enzyme, his recuperation and permit his reutilisation. There are various methods of enzyme immobilization; such as adsorption, covalent bonding, entrapment, and cross-linking. Entrapment method for enzyme immobilization keeps the enzyme trapped inside an inert and porous material without affecting its structure. Alginate is one of the polymers frequently used to do entrapment due to their mild gelling properties and nontoxicity [19].

Encapsulation of partially purified proteases from A. precatorius in alginate gel beads taking into account the effect of alginate concentration, calcium concentration, ratio of enzyme to alginate on enzyme activity, was explored in this work as contribution to the preservation of enzyme activity.

2. Materials and methods

2.1. Materials

The leaves and stems of A. precatorius were collected in Ngaoundere market, Adamawa Region (7°19′12″N, 13°35′48″E) of Cameroon, in February 2020. The partially purified extract was obtained using the method described by Ngangoum et al. (2017) [6] with slight modifications. The leaves and stems of A. precatorius were used to extract the protease and the partially purified extract was included in calcium alginate (see Figure 1).

2.2. Optimization of the immobilization parameters of partially purified proteases from A. precatorius in an alginate gel

The immobilization parameters were studied and optimized to preserve the activity of the partially purified enzymes extracted from stems and leaves of A. precatorius. The Central Composite Design (CCD) was used to study the effect of three variables (alginate concentration, CaCl2 concentration and enzyme/alginate ratio) for the entrapment of protease from A. precatorius. The experimental matrix is presented in Table 1. Seventeen tests were carried out, including three points at the center, eight factorials and six stars points. The response measured was protease activity.

2.3. Entrapment of partially purified proteases of A. precatorius in an alginate gel beads

An adapted method of Won et al. (2005) [8] with slight modifications was used. Sodium alginate solution 1–5% w/v (alginate powder with 50 mM Tris–HCl buffer (pH 8)) was mixed with partially purified protease solution to obtain different enzyme alginate ratio (10–30% v/v), then the mixture was thoroughly stirred to ensure complete blend. As soon as the mixed solution was dripped into 10 mL of CaCl2 solution (100–400 mM) using a syringe, Ca-alginate beads were formed. After 30 min of hardening, the beads were separated from the calcium chloride solution by simple straining. They were washed on a filter with distilled water and two times with 50 mM Tris–HCl buffer (pH 8). The filtered CaCl2 solution and the washing solutions were collected for loading efficiency determination.

2.4. Determination of the activity of immobilized enzyme

The method used was adapted from that of Benzian (2003) [10] with slight modifications. Hydrolytic activity of protease was assayed by spectrophotometry. To 10 mL of Tris–HCl buffer (0.5M pH 8) 0.1g of casein was added. The hydrolysis reaction was started by the addition of 3 g of the protease-entrapped beads (corresponding to the total of beads obtain for each experiment) and raising the temperature to 37 °C. Hydrolysis lasted for 30 min. Samples were withdrawn every 5 min, and tyrosine released by the reaction was monitored at 660 nm using a spectrophotometer (JENWAY 7310 manufactured in UK). Initial rates were the slopes of the tangents at the origin of the kinetics (\( \text{V}_0 = \frac{d}{dt} [\text{Tyrosine}] \)). The amount of protein in the beads was calculated, considering that the loading efficiency was 100%. The Protease Activity (PA) of the preparation was calculated using formula (1) [10]:

\[
\text{PA} = \frac{\text{V}_r \times V_e \times F}{10^3 \times V_e} \text{ (U/mL)}
\]

Where:

- \( \text{V}_0 \): initial velocity (µg. L−1. min−1),
- \( V_r \): reaction medium volume (11mL),
- \( V_e \): volume of enzyme dilution (1 mL),
- \( F \): dilution factor of enzyme preparation (1).

By analogy, according to the investigation of Loncle (1992) [11], a protease unit is defined as the amount of enzyme that can hydrolyze one microgram of casein per minute under operating conditions.

2.5. Determination of the kinetic parameters of the free and immobilized enzyme

The method used was adapted from that of Benzian (2003) [10]. In order to determine the kinetic parameters KM and Vmax of the protease, the concentration of the substrate subjected to the enzyme was varied. The initial rates (\( \text{V}_\text{in} \)) of the reactions were the slopes of the tangents at the origin of the kinetics (\( \text{V}_\text{in} = \frac{d}{dt} [\text{Tyrosine}] \)), at 37 °C. One mL of the enzyme preparation and alginate beads were successively put in the presence of different concentrations of casein diluted in Tris–HCl buffer 0.5 M pH 8 (these concentrations varied from 0.2 to 1% p/v). The absorbance was read at 660 nm.

2.6. Determination of the loading efficiency

Loading efficiency was obtained by estimating the amount of enzymatic protein retained in the calcium alginate network. After the formation of the alginate beads, the strained CaCl2 solution and washing water were collected to determine the effectiveness of the entrapment. This was determined using the Lowry method. Loading Efficiency was defined as follows [9]:

\[
\text{Loading Efficiency (\%)} = \left( \frac{C_i V_i - C_f V_f}{C_i V_i} \right) \times 100
\]

Where:

- \( C_i \) is the initial concentration of proteins,
- \( V_i \) the initial volume of enzymatic solution,
- \( C_f \) the concentration of proteins in the total strain, and
- \( V_f \) the total volume of the strain [9].

2.7. Determination of immobilization yield

Immobilization yield was used to compute the percentage of enzyme activity retained by the protease after entrapment. Immobilization yield was determined using formula (3) [9]:

\[
\text{Immobilization yield (\%)} = \frac{\text{PA}_{\text{imm}}}{\text{PA}_{\text{free}}} \times 100
\]

Where:

- \( \text{PA}_{\text{imm}} \) is the protease activity of the immobilized enzyme (U/mL) and
- \( \text{PA}_{\text{free}} \) the protease activity of the free enzyme (U/mL).

2.8. Study of the preservation of the protease activity of alginate beads

The method used was adapted from that of Benzian (2003) [10]. In order to test the retention of the activity of the protease trapped in the
3. Results and discussion

Partially purified enzymes were immobilised in alginate gel beads using a Central Composite Design. This experimental design has three factors: alginate concentration (1–5%), CaCl₂ concentration (100–400 mmol/L) and enzyme/alginate ratio (10–30%).

3.1. Results of the experiment design

The experimental and calculated responses of the 17 experiments are presented in Table 2.

This table shows that the factors studied (alginate (A), CaCl₂ concentration (B) and enzyme/alginate ratio (C)) influenced the observed experimental response. Indeed, whatever the combination of factors, different responses were obtained. It was also noticed that the experimental values were very similar to the calculated values.

![Diagram of enzyme immobilisation](Image)

**Table 1.** Experimental Matrix of the Central Composite Design for inclusion.

| No. | Coded values | Actual values |
|-----|--------------|---------------|
|     | A  | B  | C | A  | B  | C  |
| 1   | -1 | 0  | 0 | 1  | 20 | 250|
| 2   | -1 | -1 | -1| 1  | 10 | 100|
| 3   | -1 | -1 | 1 | 1  | 10 | 400|
| 4   | -1 | 1  | 1 | 1  | 30 | 400|
| 5   | -1 | 1  | -1| 1  | 30 | 100|
| 6   | 0  | 1  | 0 | 3  | 30 | 250|
| 7   | 0  | 0  | -1| 3  | 20 | 100|
| 8   | 0  | 0  | 0 | 3  | 20 | 250|
| 9   | 0  | 0  | 0 | 3  | 20 | 250|
| 10  | 0  | -1 | 0 | 3  | 10 | 250|
| 11  | 0  | 0  | 1 | 3  | 20 | 400|
| 12  | 0  | 0  | 0 | 3  | 20 | 250|
| 13  | 1  | -1 | -1| 5  | 10 | 100|
| 14  | 1  | 1  | 1 | 5  | 30 | 400|
| 15  | 1  | 1  | -1| 5  | 30 | 100|
| 16  | 1  | -1 | 1 | 5  | 10 | 400|
| 17  | 1  | 0  | 0 | 5  | 20 | 250|
Table 2: Experimental and Calculated responses.

| No | Factors | Protease activities | Experimental | Calculated |
|----|---------|---------------------|--------------|------------|
|    | A       | B       | C     |             |            |
| 1  | 1       | 10      | 100   | 0.909       | 0.867      |
| 2  | 1       | 20      | 250   | 1.250       | 1.200      |
| 3  | 1       | 10      | 400   | 1.429       | 1.516      |
| 4  | 1       | 30      | 100   | 0.876       | 0.920      |
| 5  | 1       | 30      | 400   | 0.650       | 0.602      |
| 6  | 3       | 30      | 250   | 0.602       | 0.606      |
| 7  | 3       | 10      | 250   | 0.945       | 0.831      |
| 8  | 3       | 20      | 400   | 0.640       | 0.556      |
| 9  | 3       | 20      | 250   | 0.618       | 0.781      |
| 10 | 3       | 20      | 250   | 0.710       | 0.781      |
| 11 | 3       | 20      | 250   | 0.795       | 0.781      |
| 12 | 3       | 20      | 100   | 0.708       | 0.682      |
| 13 | 5       | 30      | 100   | 1.066       | 1.006      |
| 14 | 5       | 20      | 250   | 0.848       | 0.788      |
| 15 | 5       | 10      | 100   | 0.467       | 0.542      |
| 16 | 5       | 30      | 400   | 0.043       | 0.104      |
| 17 | 5       | 10      | 400   | 0.623       | 0.607      |

Statgraphics Centurion XV. II generates a second-degree polynomial equation describing the effect of immobilization conditions on protease activity of partially purified extract of *A. precatorius*:

\[
Y = 0.340314 - 0.404129A + 0.0383818B + 0.0078576C + 0.0533732AA + 0.0051375AB - 0.0005487AC - 0.00061907BB - 0.00016115BC - 0.0000071892CC
\]

\[
Y = \text{protease activity}, \quad A = \text{alginate concentration}, \quad B = \text{enzyme/alginate ratio} \quad \text{and} \quad C = \text{CaCl}_2 \text{ concentration}
\]

The R² value (0.94) obtained shows a better correlation between experimental and predicted value.

3.2. Validation of the final model

To validate the model, R² should not be less than 80%, the AADM value should be close to zero, the Bf should be between 0.75 and 1.25. The good fitness of the second-order polynomial equation and the behavior of the system was confirmed by a coefficient of determination (R² = 0.94; R² (adjusted) = 0.87), Absolute Average Deviation (AADM = 0.156), and bias factor (Bf = 1.05 and Af = 1.13). The repetition of the experiments under the optimized conditions permit to validate the model. The predicted value was 1.52 U/mL, and the experimental value was 1.5 U/mL.

3.3. Enzyme activity, loading efficiency and immobilisation yield

3.3.1. Enzyme activity

The activity of an immobilized enzyme is that for which it’s intrinsic properties may have been altered as a result of fixation on a support and affected by reactants at different concentrations than that which reaches the soluble enzyme. In addition, all enzyme molecules are specific and the modification of intrinsic properties is more or less important from one enzyme molecule to another.

Enzyme activity was determined according to a slightly modified method described by Mezajoug et al., (2015) [12]. Protease activity obtained are 20.86 ± 0.018 and 23.6 ± 0.026 U/mL for the crude and the partially purified extract respectively. The partial purification step improved the enzymatic activity of proteases from the leaves and stems of *A. precatorius*. These values result in the 1.2-fold concentration of enzymatic proteins and the elimination of some compounds during partial purification. These compounds may be fibers and secondary metabolites (phenolic compounds, oxalates, protease inhibitors) which may interfere with the activity of proteolytic enzymes thus affect protease activity. Protease activity was also determined by the method described by Benzian (2003) [10]. The protease activity of free enzyme was 1.78 U/mL and the combination of optimal point factors made it possible to measure a protease activity of 1.5 U/mL for the immobilised protease. The main phenomena causing this decrease in protease activity are: modification of the three-dimensional structure of the enzyme under the action of stress due to fixation, modification of local pH, electrostatic interactions, diffusional stresses, and steric clutter. These changes play a decisive role in the activity and stability of the enzyme [13].

3.3.2. Loading efficiency

Loading efficiency enabled the estimation of the percentage of enzyme proteins retained in the calcium alginate network. This measurement was made at the optimum point (1%; 10%; 400 mmol/L). The percentage of enzymes retained in the beads at the optimum point is 67%. More than 50% of the enzyme was retained in the network. Entrapment in calcium alginate is therefore effective in trapping enough enzyme in partial purified extract from *A. precatorius*.

3.3.3. Immobilization yield

The immobilization performance is defined as the relationship between the protease activity of the immobilized enzyme and that of the free enzyme.

The diameter of the beads was 3±1mm. The protease activity of immobilized enzymes is smaller (0.04–1.42 IU/mL) compared to those of free enzyme (1.78 IU/mL). The immobilization yield was 84.07%, indicating a loss of 15.93% of protease activity. The purified *Labeo rohita* visceral protease was immobilized in a 2% solution of sodium alginate and gave a maximum entrapped activity of 48.31%. The immobilization yield of the entrapment of purified *Labeo rohita* visceral protease was lower because the alginate concentration was high, compared to the alginate concentration used for the entrapment of *A. precatorius* protease (1%) [14]. For the optimisation of Immobilisation Condition of Protease extracted from Silver Catfish (*Pangasius sutchi*) Viscera in Calcium Alginate by using Response Surface Methodology (RSM), the highest proteolytic activity was obtained using sodium alginate and calcium chloride solution concentrations at 3.00% (w/v) and 0.30 M, respectively [20]. Figure 2 shows alginate beads in which partially purified *A. precatorius* enzymes were entrapped.

![Figure 2](image-url)
Figure 3. Effects of alginate, enzyme/alginate ratio and CaCl₂ concentration on protease activity.

Figure 4. Effects of interactions of different factors on protease activity. (+) high level of the setting; (-) low level of the setting; AB: interaction of alginate - enzyme/alginate ratio; AC: interaction of alginate - CaCl₂ concentration; BC: interaction of enzyme/alginate ratio - CaCl₂ concentration.

Figure 5. Effect of alginate - CaCl₂ concentration (AC) on protease activity. CaCl₂: CaCl₂ Concentration (mmol/L), ALG: Alginate (%).
3.4. Effects of immobilization conditions on protease activity

The effects of alginate, enzyme/alginate ratio and CaCl₂ concentration on protease activity are presented on Figure 3. Figure 3 shows that protease activity decreased proportionately with alginate concentration. An increase in sodium alginate concentrations causes extensive cross-links between network strings of the matrix, resulting in the reduction of pore size of beads and reduction of mass transfer or diffusion of substrate to the enzyme active site [15].

The results showed that protease activity decreased when enzyme/alginate ratio increased. The large amount of protease molecules led to steric hindrance at the active sites [10].

When CaCl₂ concentration increased from 100 mmol/L to 200 mmol/L, protease activity also increased. According to the "Egg-box" model, divalent cations selectively bind to blocks guluronates from the alginate chain to form dimers. When the calcium concentration is large enough, there is an association of dimers to form multimers [16]. These associations allow the creation of an open pore structure with high porosity within the crosslinked alginate solution. Above 200 mmol/L, protease activity decreased. The use of a higher concentration of divalent cations caused the formation of a more compact gel. This leads to a decrease in pore sizes and therefore limits protein diffusion. This permitted observing that each factor has an effect on the preservation of protease activity.

From the interaction plots, some of the lines do not cross (1). It can also be seen that some of the lines are completely intersecting, indicating that there is an interaction between alginate concentration and CaCl₂ concentration, also between enzyme/alginate ratio and CaCl₂ concentration. The interaction of alginate - CaCl₂ concentration (2) and enzyme/alginate ratio - CaCl₂ concentration (3) significantly influence the protease activity of the immobilized enzyme. Indeed, the variations in alginate content have cross-effects with that of CaCl₂ concentration, in enzyme/alginate ratio and CaCl₂ concentration respectively.

The representations on Figures 5 and 6 could help to a better understanding of each interaction.

Figure 5 shows the protease activity versus alginate-CaCl₂ concentration. It appears that when CaCl₂ concentration was at its lowest level (100 mmol/L), the protease activity decreased, with the increase of alginate concentration. If the alginate concentration increases, no matter the concentration in CaCl₂, the pore diameter will smaller. When CaCl₂ concentration is at its high level (400 mmol/L), protease activity decreased as alginate content increased. This showed an interdependence between alginate concentration and CaCl₂ concentration.

The effect of enzyme/alginate ratio and CaCl₂ concentration is shown in Figure 6.

This figure indicates that when CaCl₂ concentration was at its lowest level (100 mmol/L), protease activity increased as the enzyme/alginate ratio increased. This means that the concentration in enzymes in the solution entrapped positively affect the protease activity if CaCl₂ concentration is low. When CaCl₂ concentration was at its highest level (400 mmol/L), protease activity decreased as the enzyme/alginate ratio increased. When alginate concentration increase, the viscosity of the solution (sodium alginate + Tris–HCl buffer) is improved [17]. The transmission of water molecules is hampered, which leads to a decrease in crystal size. Therefore, the pore diameter is negatively correlated to the alginate concentration, which explain the decrease of enzyme activity.

3.5. Use of the immobilized beads for several cycles of hydrolysis

The beads obtain at the optimal point was used to test the reuse of the immobilized protease. The hydrolysis of casein by the A. precatorius protease immobilized in alginate beads was test for 5 cycles. Figure 7 present the evolution of protease activity within five days of storage at 4 °C and 26 °C.

The immobilized enzyme is reusable for around 4 days. The storage at 26 °C show better conservation of this activity from the second to the

![Figure 6. Effect of Enzyme/alginate ratio - CaCl₂ concentration (BC) on protease activity. CaCl₂: CaCl₂ Concentration (mmol/L).](image)

![Figure 7. Repeated use of immobilized protease.](image)
fourth day. The free protease (purified) is stable throughout a wide temperature range from 10 to 80 °C [7]. This shows that the immobilized protease is more stable at 26 °C than at 4 °C. The second day, the immobilized catalyst has already lost around 50% of its activity. Repeated use brought about activity loss in all cases. This might be mainly due to enzyme leakage from the alginate gel beads. Another possible explanation is damage of the alginate beads during repeated use. However, as they were reused, protease entrapped and store at 26 °C retained higher activity than the protease entrapped and store at 4 °C [9].

3.6. Initial velocity of free enzyme and immobilized enzyme

The initial linear rate of product formation also called the initial velocity (Vin or V0) has been found by plotting the graph of product concentration as a function of time and measuring the slope at time zero. It is one of the most important characteristics of any enzyme-catalyzed reaction. One factor that affects the initial velocity is the substrate concentration (casein μg/mL). Figure 8 shows the initial velocity patterns of the free enzyme and the immobilized enzyme based on casein concentration used as substrate.

The concentration of the substrate subjected to the enzyme varied and for each concentration, the initial velocity is the slope of the curve tangent of substrate appearance in the medium. There was an increasing difference in the velocity of reaction as substrate concentration increases to a stable ratio of 6:1 (the average of the ratio of two velocities at the same casein concentration on the curve of the free and those of immobilized enzyme 120/30; 140/23). The pH and temperature that affect the reaction velocity were constant (pH 8, 37 °C) and the same for both reactions. Increasing the substrate concentration induces an increase in velocity in both cases. This means that the steric hindrance factor would be primarily responsible for the great difference observed. Indeed, the entrapment of enzyme by alginate greatly reduces access of the substrate to the active sites because the enzyme is inside an inert and porous material [15].

3.7. Effects of immobilization on hydrolytic capacity of A. precatorius partially purified extract

To visualize the effect of immobilization on hydrolysis capacity of A. precatorius partially purified extract, a casein hydrolysis kinetic was followed by measuring the amount of tyrosine released with respect of the hydrolysis time. Tyrosine is a C-terminal amino acid of casein. The amount of tyrosine present in the solution will provide information on the progression of hydrolysis. The curves in Figure 9 shows the amount of tyrosine released over time.

The figure shows that the rate of the product released (tyrosine), is significantly lower for the immobilized enzyme compared to the free enzyme, by a factor of 1:3. In fact, in both cases, when the enzyme is put in contact with the substrate, the reaction started immediately but after 5 min, the amount of tyrosine release is about 120 μg for the free enzyme and 40 μg for the immobilized one. This is because steric clutter limits the amount of substrate that reaches the active site of the immobilized enzyme and as consequence, the product is not well liberated. It was also found that the two curves have the same shape. Therefore, immobilization of the enzymes does not prevent the reaction from taking place nor change the mechanism of the substrate hydrolysis, it only slowdown the reaction.

3.8. Maximum velocity (Vmax) and Michaelis-Menten (KM) constant of free and immobilized enzymes

The Michaelis Menten's constant Km is a kinetic parameter which helps to characterize an enzymatic reaction and to evaluate the enzyme-substrate affinity. Km is the substrate concentration for which the enzyme is at half saturation. High affinity is indicated by a small Km, meaning that the reaction rate will be at its maximum Vmax quickly. The
value of KM depends on the enzyme, the substrate, the temperature and the pH [18]. The Lineweaver-Burk representations of free and immobilized enzymes are shown on Figure 10.

Free protease of *A. precatorius*, with a Vmax of 153.85 g.L⁻¹ min⁻¹ hydrolysed casein faster than the immobilized one that has a Vmax of 46.95 g.L⁻¹ min⁻¹ on the same substrate. Since the Vmax of the immobilized enzyme is far from that of the free enzyme, it means that the catalytic activity has been slowed down [10].

With a KM of 1.26 mg/mL, the free protease of *A. precatorius* has a higher affinity for casein compared to that immobilized (6.83 mg/mL). The KM increased considerably due to the establishment of a substrate concentration gradient inside the beads. When the support and the substrate are electrically charged, attractive or repellant electrostatic interactions are created in the immediate environment of the enzyme, resulting in the establishment of a substrate concentration gradient and a change in the apparent affinity of the enzyme for its substrate. KM increases if the charges of the substrate and the support have the same sign; and decreases if the charges are of opposite signs [10].

The difference in reactivity could be explained by the fact that the immobilization of enzymes affects their kinetic properties. Thus, in the case of immobilization of *A. precatorius* protease in calcium alginate gel beads, diffusion phenomena limit the access of substrate (casein) to enzymatic sites [13].

4. Conclusion

The activity of the enzyme was successfully preserved (84.07%). Effects of immobilization conditions (alginic acid content, enzyme/alginate ratio, and CaCl₂ concentration) on enzyme activity were investigated. Free enzyme hydrolysed 3 time faster than immobilized one and the optimum activity was obtained for an alginate content of 1% (p/v), an enzyme/alginate ratio of 10% (v/v) and a CaCl₂ concentration of 400 mmol/L. 67% of protein was retained in the beads at the optimum point. Among the tested parameters, alginate concentration and enzyme/alginate ratio were more important for protease entrapment in Ca-alginate beads. At the optimum, kinetic parameters of the immobilized enzyme (KM (6.83 mg mL⁻¹) and Vmax (46.95 g.L⁻¹ min⁻¹) were comparable to those of free enzyme, KM (1.26 mg mL⁻¹) and Vmax (153.85 g.L⁻¹ min⁻¹). Immobilization process reduce the affinity of the enzyme for the substrate. Once immobilized, these enzymes are more stable and easier to store. Immobilized enzymes can be easily separated from the substrate and the product which allows better control of the process. It would be interesting to reuse them several times, in batch reactors or in continuous processes, studying the evolution of the activity over a long period. Their substrates could be the defatted flours of some local oleo protein crops (soya, *Ricinodindron heudelotii*, peanut) to obtain protein hydrolysates having functional and nutritional properties of particular interest for the food, pharmaceutical and cosmetic industries.

**Declarations**

**Author contribution statement**

Wadjji Djenkwe Nina Vanessa: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Mezajoug Kenfack Laurette Blandine: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Nse Emmanuel Jong: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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**Data availability statement**

Data will be made available on request.

**Declaration of interests statement**

The authors declare no conflict of interest.

**Additional information**

No additional information is available for this paper.

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