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Article

Covalently entrapping catalase into calcium alginate worm pieces using EDC carbodiimide as a crosslinker

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SUMMARY

Catalase is a biocatalyst used to break down toxic hydrogen peroxide into water and oxygen in industries such as cheese and textiles. Improving the efficiency of catalase would help us to make some industrial products, such as cheese, less expensively. The best way to maintain catalase's conformation, and thus enhance its activity, is to immobilize it. The primary goal of this study was to find a new way of immobilizing catalase. There are many ways to immobilize an enzyme, one of the foremost being entrapment in calcium alginate. In past literature, researchers used calcium alginate as a bead, and an enzyme was physically entrapped into it. This form of immobilization has been proven inefficient because the enzyme is loosely attached to the material. To address this issue, we used calcium alginate as worm pieces with a carbodiimide as a crosslinker. Worm pieces here refer to small uniform pieces of cylindrical calcium alginate made by cutting long strings (worms) into small pieces. This research set out to understand whether or not EDC(1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) can increase the immobilization of catalase into the calcium alginate. We found that EDC is able to triple the amount of catalase immobilized in calcium alginate compared to only calcium alginate entrapment. This research devised a new type of covalent entrapment that can be used to immobilize many other enzymes that also contain lysine residues. The result suggests that catalase can be better covalently entrapped into the calcium alginate worm pieces using EDC.

INTRODUCTION

Catalase (EC 1.11.1.6) is an enzyme that is found in nearly all living organisms. The main activity of catalase is to break down toxic hydrogen peroxide into simple water and oxygen. This activity of catalase helps the cell to main its homeostasis by reducing the number of reactive oxygen species (ROS). Catalase also has a variety of potential uses in the medical industry, food industry, bioremediation, and much more (1). One of the popular uses of catalase is to remove hydrogen peroxide (H_2O_2) from milk to produce cheese, because H_2O_2 is toxic to the bacteria that help in cheese production (1). Bioremediation is a process of treating pollutants from the environment using biological specimens. Catalase can also be used to bioremediate H_2O_2 from bleached textile effluents that are not treated well (1).

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Catalase must be immobilized into some material if it needs to be used in an industrial setting because this enhances its activity. There are many ways to immobilize catalase, including inorganic supports, natural polymers, and synthetic polymers (2). The primary goal of immobilization is to increase the efficiency of the enzyme. This is important because it helps to promote the enzyme's activity so the industrial company can successfully use it to increase the speed of a chemical reaction.

Calcium alginate is one of the natural polymers popularly used for both cell immobilization and enzyme immobilization (3). Alginate is found in algae, and through an industrial process it is commercially purified and sold (3). When sodium alginate (NaAlg) is placed in a divalent solution (Group 2 elements), the carboxylate groups in the alginate get crosslinked with the divalent ions, allowing the catalase to be entrapped into the spaces (2). The sodium in the alginate gets displaced by the calcium ions once it is placed in calcium chloride solution (3). The final material calcium alginate is a hard structure that is easy to handle.

To immobilize catalase using alginate, carbodiimide chemistry can be used. In this chemical reaction, EDC(1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride), a carbodiimide that can crosslink carboxylates to primary amines, is used to crosslink proteins with the immobilizing material (4). Catalase and most other enzymes have lysine residues in their amino acid composition. Since the lysine group (NH2) holds a positive charge, it is found mainly on the surface of a protein. This property allows for easy crosslinking without denaturing the enzyme (4). Sodium alginate is composed of multiple carboxylates which can be used for crosslinking. EDC is first allowed to activate these carboxylate groups in an aqueous solution and then the protein containing lysine residues is added to the solution for effective crosslinking between the carboxylates and the protein's lysine residues (4). After the crosslinking, isourea is released as a byproduct (4). We employed this carbodiimide chemistry in this study between alginate and catalase.

Traditionally, calcium alginate has been used as a bead to immobilize enzymes and cells (5). In contrast, this study uses calcium alginate worm pieces. Worm pieces are made by cutting the calcium alginate strings (worms) into small pieces. Worm pieces are effective because they have a better surface area to volume ratio than beads. We hypothesized

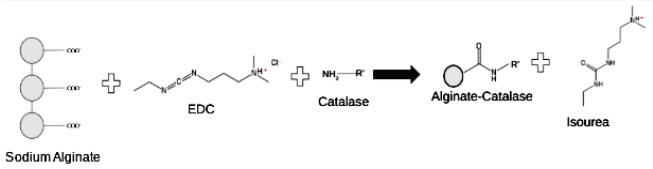

Figure 1: Mechanism of crosslinking using EDC. The carboxylate group of alginate is first activated by EDC (not shown in the figure). Once the carboxylate is activated, the catalase with a lysine residue is added to crosslink with the carboxylate. After the reaction, isourea is released as a byproduct. Isourea is removed from the mixture when it is placed in a calcium chloride solution. The structures were designed using PubChem Sketcher V2.4.

that catalase activity would increase drastically when the calcium alginate worm pieces are crosslinked to catalase with EDC carbodiimide because EDC has the ability to form a covalent bond between the carboxylate group in the alginate and lysine residue group in the catalase. Due to this, the enzyme can firmly attach to the material.

RESULTS

The following observations were made by immobilizing catalase in calcium alginate under various conditions. In order to first determine the molarity of the 3% H_2O_2, we first measured the amount of H_2O_2 present in a control sample containing 3% H_2O_2 by titrating with an oxidizing agent and monitoring the color change. The molarity of the solution was found to be 0.9 M. In order to understand the baseline activity of catalase, free catalase was added to an H_2O_2sample solution. In order to measure the catalase's activity, we measured the amount of H_2O_2 remaining after a specific period of time in the 0.9 M sample by titrating it with KMnO4. This showed an activity of 1182 IU. We then prepared catalase immobilized in calcium alginate worm pieces, either with or without EDC (**Figure 1**). Sodium alginate showed the lowest activity after immersion in calcium chloride solution for 30 minutes and showed the highest activity after immersion for 15 minutes (**Table 1**).

The next step was to check whether EDC improved the activity. Adding EDC to give a final concentration of 10 mM increased the activity of catalase three-fold compared to calcium alginate alone (**Table 2**). When the calcium alginate worm pieces with and without EDC were compared, the calcium alginate with EDC showed the highest activity (**Figure 2**). This result supports the hypothesis that calcium alginate with EDC carbodiimide is a better candidate for catalase immobilization.

DISCUSSION

The main goal of this study was to effectively immobilize catalase into calcium alginate worm pieces by using EDC carbodiimide. When EDC was used to activate the carboxylate group in NaAlg, the catalyst's activity was higher than in calcium alginate without EDC. We observed the highest activity when EDC was dissolved to a final concentration of 10 mM. This might be due to the fact that there was more EDC to activate carboxylate groups in 10 mM EDC than in 4 mM EDC.

H_2O_2 (control)	0 IU
Catalase-H_2O_2	1182 IU
Calcium Alginate 2%, 15 min	251 IU
Calcium Alginate 2%, 30 min	130 IU

Table 1: Catalase activity in each sample with 20 µL undiluted catalase solution

Calcium Alginate 2%, 30 min	IU Immobilized
4 mM EDC	282 IU
10 mM EDC	399 IU

Table 2: The activity of catalase immobilized in calcium alginate using EDC

The catalase was able to be successfully immobilized both by entrapment and by covalent linkage in calcium alginate. The reason why immobilized catalase had less activity than free catalase might be due to enzyme activity loss during immobilization.

This study developed a new way of immobilizing an enzyme that is more efficient and easier to handle than regular calcium alginate beads. This result may lead to further research on enzyme immobilization. In the future, research can be done to find out whether the same technology can be used to immobilize many other enzymes that also contain lysine residues in their nonactive sites.

There are many applications of this technology in the manufacturing sector, including the cheese industry. It is widely accepted that hydrogen peroxide halts the growth of the bacteria in the milk that is essential for cheese production. We can use the immobilized catalase to break down this toxic hydrogen peroxide. The same immobilized catalase can also be used in the textile industry to remove peroxides from fabric. The long-term outcome of this study would be to develop a universal, cost-effective way to immobilize enzymes.

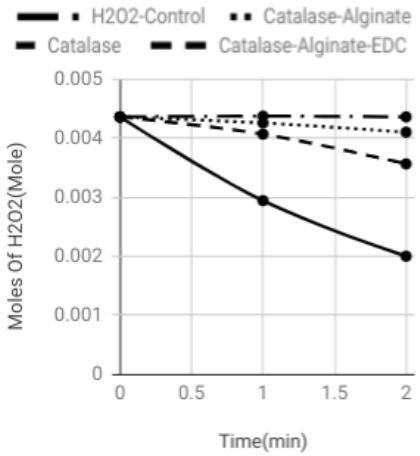

Figure 2: The activity of catalase for 2% NaAlg- 30 minutes. The graph shows the moles of H2O2 present in the sample after a t=0,1 and 2 minutes. The graph compares the moles of H2O2 remaining in a 5.0mL sample with no catalase, free catalase, immobilized catalase without EDC and immobilized catalase with EDC.

MATERIALS AND METHODS

Sodium Alginate And Calcium Chloride Solution

The 2% sodium alginate solution was made by dissolving 2.00 g NaAlg in 100.0 mL phosphate buffer (0.1M, pH 7.0) overnight. The 0.5 M calcium chloride solution was made by dissolving 27.745 g in 500.0 mL distilled water. All these solutions were stored at room temperature for future use.

Generation of CaAlg and catalase-containing worms without EDC

In a beaker, 20.0 mL of 2% NaAlg was added and then 5720 IU of catalase was added to it. 5.0 mL was loaded into a 10.0 mL syringe and made into string-like structures called worms by dispensing into the 0.5 M calcium chloride solution. The sodium alginate with catalase was incubated in calcium chloride solution separately in two different time periods-15 and 30 minutes. The sodium in alginate is displaced by calcium and gives it a hard structure. After the time, the CaAlg was made into worm pieces with a diameter of about 2-4 mm and length of about 1-2 cm. The activity was determined by placing the pieces in 5.0 mL H_2O_2 solution and titrating after 1 and 2 minutes.

Generation of CaAlg and catalase-containing worms with EDC

To the 20.0 mL 2% NaAlg, EDC was added to give final concentrations of 4 mM and 10 mM. It was allowed to react for 15 minutes, after which 5720 IU of catalase were added. Then 5.0 mL of this solution was made like worms in a 0.5 M calcium chloride solution. It was in the calcium chloride solution for 30 minutes. The activity was measured using the same protocol as described for calcium alginate without EDC.

Determination of H2O2 concentration and measurement of catalase activity

H_2O_2 concentration was determined by titrating the sample with potassium permanganate. For ~5.0 mL H_2O_2, 12.0-13.0 mL sulfuric acid was added to give a source of hydrogen ions. 2% $KMnO_4$ was loaded into a 50.0 mL burette, then the sample H_2O_2 was titrated until the color of the sample H2O2 solution changed to light pink. The light pink marked the endpoint of titration for H_2O_2. The volume of $KMnO_4$ consumed was used to determine the moles of H_2O_2 using the following equation:

$$5H_2O_2 + 2KMnO_4 + 3H_2SO_4 \rightarrow$$
$$5O_2 + 2MnSO_4 + K_2SO_4 + 8H_2O$$

The catalase activity was determined indirectly by measuring the amount of H_2O_2 decomposed over time.

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