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Research article

Investigation of some properties of immobilized urease from *Cicer arietinum* and its using in determination of urea level in some animal feed

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Key words: Urease, alginate, immobilization, *Cicer arietinum.*

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Abstract

In this study, urease was isolated from *Cicer arietinum* and immobilized in calcium alginate beads. Various parameters, such as effect of thermal stability, temperature, optimum pH and pH stability, substrate concentration, reuse and storage stability were investigated and the results of the investigation were compared with the soluble enzyme. The activity yield of immobilization was calculated as 88.5 %. Optimum temperature and pH were found to be similar for both soluble and immobilized enzymes. It was observed that immobilization did not change pH and temperature prompt of the enzyme. pH and optimum temperature were found to be 7.0 and 50°C respectively. Improved thermal and pH stability of urease were achieved by immobilization. The K_m value for immobilized urease was found to be higher than that of the free enzyme. Immobilization of beads at optimum conditions enabled up to 5 repeated use of enzyme and maintained 58% of their initial activity. It was found that storage stability of immobilized enzyme was better than that of the free enzyme. Immobilized urease enzyme was tested for the determination of urea amounts in various animal feeds.

Introduction

Enzymes have increasing importance in industry and in medical and clinical applications. It is important as a catalyst in chemical processes. For many industrial applications, there is a demanding interest for the immobilization of the enzymes on a water soluble carrier. The immobilization of enzymes has the advantages of enhanced reuse capacity, increased pH and thermal stability as well as it enables easier separation of catalyst from the reaction mixture and makes enzymatic applications suitable for automated continuous processes [1].

Immobilization of enzymes can be carried out by different methods. Among the various techniques for enzyme immobilization, calcium alginate gel has been one of the most used matrices for enzyme entrapment due to its simplicity, inexpensiveness and non-toxicity [2]. Alginate immobilization of enzymes was used in many studies [3-5].

Hydrolysis of urea to carbon dioxide and ammonia is catalysed by a nickel dependent metalloenzyme called urease (EC 3.5.1.5, urea aminohydolase) [6]. Isolation of urease can be achieved from a wide variety of organisms including fungi, plants and bacteria. Its primarily used by these organisms to utilize urea as a nitrogen source. Plant and microbial ureases show diverse biological properties such as the activation of blood platelets, antifungal activity and insecticidal activity. These findings reinforce the hypothesis that ureases might be involved in plant defence mechanisms [7]. Urease positive bacteria and fungi, such as *H. pylori*, *Y. entereolitica, C. neoformans* play a critical role in the pathogenesis of animal and human diseases [8].

There has been increasing interest in urease enzyme in biotechnological research. The dialysis regeneration systems of artifical kidney machines and the direct removal of urea from blood for detoxification are obtained by immobilized urease applications [9]. Immobilized urease has also been used for the removal of urea from beverages and foods and for the convertion of urea present in fertilizer waste water effluents in bioreactors to ammonia and carbon dioxide [10].

Latterly, an increasing interest in the use of various natural polymers as a support material for immobilization of urease was risen [11-13]. In this study, urease was isolated from *Cicer arietinum* and immobilized in calcium alginate beads for the first time in literature. Various parameters, such as temperature effect, thermal stability, optimum pH and pH stability, substrate concentration, reuse and storage stability were investigated and the comparison of these findings with the soluble enzyme is performed. In addition, suitability of immobilized enzymes for the determination of urea in animal feed were investigated.

Experimental

Materials

Cicer arietinum samples were obtained from Edirne Agricultural Research Institute. Urease enzyme was isolated from chickpea. Sodyum alginate was purcased from Fluka Biochemica; $CaCl_2$ was obtained from Sigma. Urea was

purcased from Merck. All other chemicals were analytical grade and used without further purification.

Enzyme extraction

Cicer arietinum were broken with blender (Waring brand). Chickpea were treated with pH 7.0 Tris HCl buffer for 24 h at 4°C. Contents were mixed by magnetic stirrer for 1 h. The extract was filtered trough a Whatman filter Paper No.1. The filtrate was centrifuged for 15 min. at 12000 rpm. The supernatant was used as the crude enzyme.

Enzyme assay

Nessler ammonia assay method was used for the determination of the urease activity of both soluble and immobilized enzyme [14]. In our study, urea solution (1% in 50 mM Tris HCl buffer, pH 7.0) was used as a substrate. Reaction mixture was prepared by using 1mL of free enzyme or 0.5 g immobilized enzyme, 2.5 mL urea and 2.5 mL Tris HCl buffer. The mixture was incubated at 50°C for 40 min. Then, 100 µL Nessler reagent was added into the mixture. The absorbance was measured at 425nm. The slope of the ammonium calibration curve was used in order to calculate enzyme activity. One-unit enzyme is defined as the amount of enzyme which hydrolyses 1µmol of ammonia in 1 min. All measurements were performed at least 3 replicates and the expressed as average of the measurements.

Enzyme immobilization

Mixture of an equal volume of urease enzyme solution and sodium alginate solution were prepared to give a 3% (w/v) final concentration of sodium alginate solution in mixture. The mixture obtained was extruded drop wise through a syringe into a gently stirred 3% (w/v) $CaCl_2$ solution. It was kept at 4°C for 50 min in buffer solution until maturation. Enzyme containing calcium alginate beads were separated from the $CaCl_2$ solution by filtration. The mature beads were washed in the cold Tris HCl buffer (50 mM, pH 7.0). Bradford method was used for the protein content determination of free enzyme and washing water. Immobilization efficiency was defined as follows:

Immobilization efficiency (%) = aimm / afree x 100

aimm: specific activity of immobilized enzyme (U / mg protein) afree: specific activity of free enzyme (U / mg protein)

The effect of pH and pH stability on enzyme activity

The pH range of 6.0 – 9.0 was used for the investigation of the effect of pH on the activity of the free and the immobilized urease. The pH stability of the free and immobilized urease was determined by incubating in substrate free different buffers (pH 6.0-9.0) for 30 min at 4°C. The activity and pH stability were determined at the end of this period.

The effect of temperature and thermal stability on enzyme activity

The effect of temperature on the activities of free and immobilized urease was studied at different temperartures between 30 and 60°C. In order to investigate the thermal stabilities of the enzyme in buffer without substrate (pH 7.0 Tris HCl), free and immobilized enzyme samples were incubated for 10, 30, 60 min at varying temperatures between 40 and 60°C.

The effect of substrat concentration on enzyme activity

The effect of substrat concentration on the activity of enzyme was examined. Various concentrations (0.08, 0.16, 0.24, 0.32, 0.40, 0.48, 0.56, 0.64, 0.72, 0.82 mmol) of urea were used as substrate for urease activity assay. K_m and V_{max} values from the Lineweaver-Burk plots were calculated.

Repeated use of urease immobilized in the alginate beads

The reuse capacity of immobilized enzyme was investigated by performing repeated assays with the beads for several times. After each urease activity assay of the beads was performed, the beads were rinsed with distilled water. Then, the beads were reassayed for urease activity and the same steps were repeated.

Storage stability

The activities of the both free and immobilized urease at 4°C were measured at regular time intervals. The enzyme activities of free and immobilized urease were compared.

Assay of urea amounts in various provenders

It was investigated that the usage of immobilized chickpea urease in which for different types of provenders for the evaluation of urea amounts. Each provender contains 10 mg/kg urea. Provenders represented with initials A, B, C, D. Each sample which included 10 mg urea were distilled in Tris HCl buffer (50 mM, pH 7.0) and were centrifuged. In order to evaluate urea amounts, following table and formula were used. Standard and sample tubes were prepared and treated with Nessler reagent. Absorbance values which obtained from each groups were calculated with following formula.

$$\text{The amount of urea (mg)} = \frac{\text{Absorbace of sample x Concentration of standard}}{\text{Absorbance of standard}}$$

Results and Discussion

Immobilization of enzymes protect the stability of the high cost enzymes and enables reuse [15]. Therefore, there has been an increasing interest in use of immobilization of enzymes. Entrapment, an immobilization method, can be defined as physical restriction of enzyme within a confined network or space. Various natural or synthetic materials were used as carriers for immobilization of urease. In recent

years, urease has been entrapped with tube membranes used for therapeutic and technic purposes. Alginate, carragenan and chitosan were known as natural polymers and they have been increasingly used in enzyme entrapment studies [16, 17]. Sodium alginate is a naturally occuring copolymer consisting of mannuronic acid and gluronic acid. Due to the cost effectiveness of sodium alginate, it is widely used for entrapment enzymes. High gel porosity and relatively inert aqueous environment within the alginate matrix provides high diffusion rates of macromolecules. In this study, urease was isolated *from Cicer arietinum* and immobilized in calcium alginate beads.

The effect of pH and pH stability on enzyme activity

pH is an important parameter affecting the enzyme activity. The optimum pH of the urease in earlier studies reported as values changes between 4.5-9.0 [18-21]. In this study, optimum pH for both enzymes was determined as pH 7.0. It was observed that the optimum pH did not show any changes after immobilization (Figure 1).

pH stability of free and immobilized urease was determined by incubating in different buffers (pH 6-9) for 40 min at 50°C. Stability retaining a considerable amount of activity at higher and lower pH values improved when compared with the free urease and immobilized urease (Figure 2 and Figure 3).

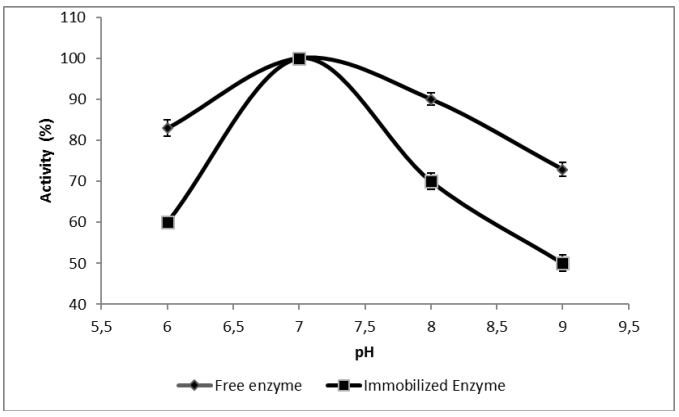

Figure 1. The effect of pH on free and immobilized urease activity

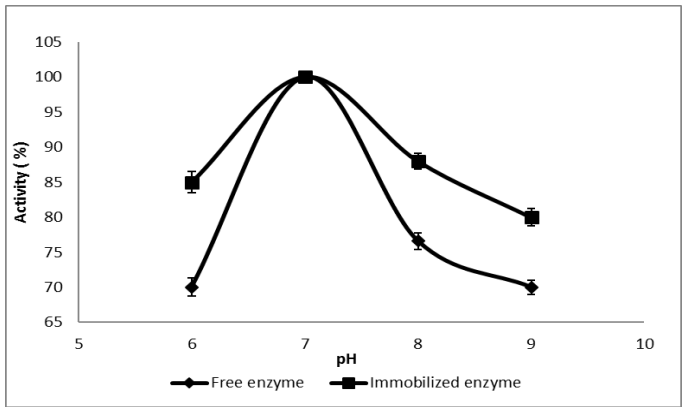

Figure 2. The effect of pH stability on free and immobilized urease activity

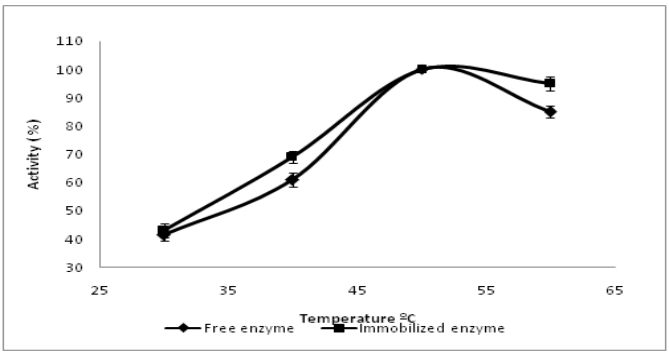

Figure 3. The effect of temperature on free and immobilized urease activity

The effect of temperature and thermal stability on enzyme activity

The changes in the optimum temperature depends on the source of enzyme [18, 22, 23]. As an example, the optimum temperature for nut urease indicated as 25 ° C [24]. Our study showed that optimum temperature of free and immobilized urease is 50°C (Figure 3). In this study, thermal stabilities of free and immobilized urease were studied at different time and temperature without substrate. Both enzyme forms retained their initial total activities at 40°C-50°C after 60 min. Immobilized enzyme retained 92% of its initial activity after 30 min at 60°C, whereas the free enzyme retained 63% of its activity. After incubating for 60 minutes at the same temperature free enzyme lost about 60% of its initial activity, whereas the immobilized enzyme lost about 35% of its initial activity (Figure 4 and Figure 5).

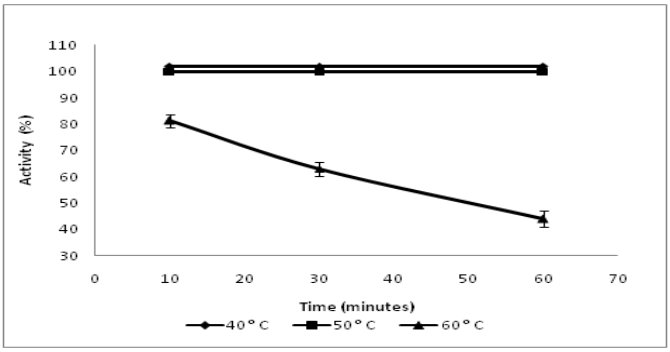

Figure 4. The effect of temperature on stability of free urease

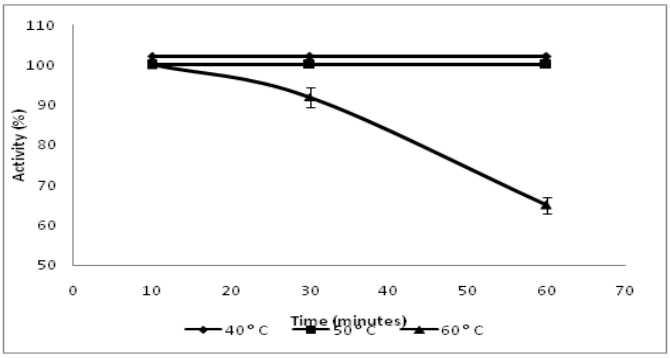

Figure 5. The effect of temperature on stability of immobilized urease

As immobilization protects the enzyme from environmental factors and preserves the tertiary structure of the enzyme, it is believed that immobilization increases the thermal stability. Generally, the activity of the immobilized enzyme is more stable than free enzyme againts temperature and denaturing agents [25, 26].

The effect of substrat concentration on enzyme activity

Measurement of enzyme activity at varying concentrations of urea was performed in order to determine K_m and V_{max} values of free and immobilized enzyme. Free and immobilized enzyme K_m values were found to be 0.42 mM and 1.33 mM, respectively. The V_{max} of immobilized enzyme increased two fold than that of the free enzyme (Figure 6).

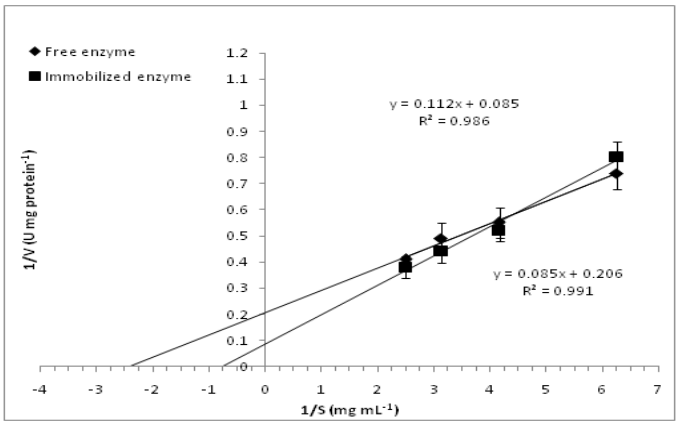

Figure 6. The effect of substrate concentration on free and immobilized urease activity

Repeated use of immobilized enzyme

Free form of the enzyme did not show any capacity to be used again. Thus, enzymes in the industrial field can used repeatedly used after they are immobilized [27]. In our study, chickpea urease immobilized by alginate were used repeatedly up to 5 times. Also, immobilized enzyme retained 60% of its initial activity at the end of the five cycles (Figure 7).

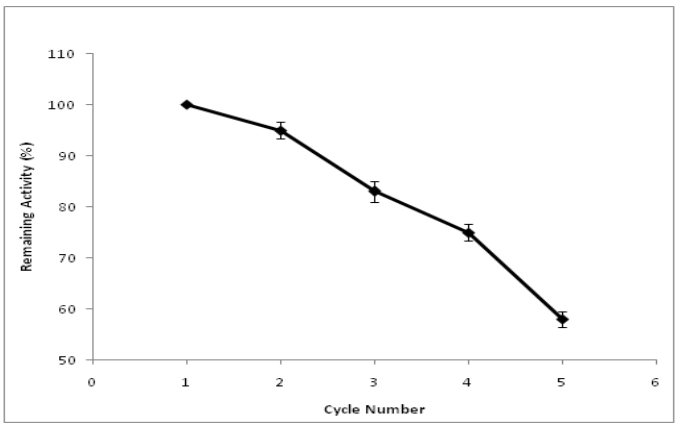

Figure 7. Repeated use of immobilized enzyme

Storage stability

Immobilized and free enzyme samples stored at + 4 ° C and storage stability was investigated by measuring the activity once every 24 hours. Immobilized enzyme preserved 100% of its initial activity for 3 days. Free enzyme lost approximately 40% of its initial activity within 2 days. Almost all of the activity of the free enzyme lost at fourth day. It is observed that the immobilized enzyme retained 40% of its activity after 7 days (Figure 8).

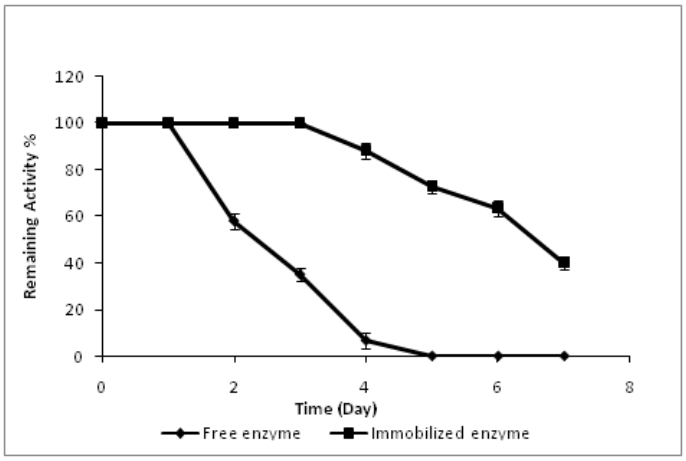

Figure 8. Storage stability

Assay of urea amounts in various provenders

Due to presence of positive effect on body weight and supplemental activitiy on proteins, urea can be present in provenders. Some studies showed that, excessive urea levels can have toxic activity [28-30]. Therefore, determination of the urea level in provenders is critical. In our study, we used two lactic provenders and two zoic provenders. As per ingredients, the provenders were represented as initials A, B, C, D. The level of urea in each provenders were determined by immobilized chickpea urease enzyme. The urea levels in the provenders which theoretically 10 mg urea were calculates as values in Table 1. Our present study showed that immobilized chickpea urease allowed precise measurement of the urea levels in provenders. The results were significantly correlated with the theoretical urea levels. In conclusion, with the optimization of the measurement condition and methods, chickpea urease can be used in provender industry for measurement of the urea level. Further studies are needed to evaluate to which extent this method could enhance current determination of urea levels in the industrial area.

Conclusion

In the present study, urease was isolated from *Cicer arietinum* and immobilized in calcium alginate beads. Immobilized urease has certain advantages compared to the free urease such as thermal stability, pH stability and storage stability and reuse. In addition, alginate has advantages over other materials because of its easy preparation procedure

and low cost. Chickpea urease showed very good entrapment in alginate. The activity yield of immobilization was 88.5 %. In addition, it is figured out that the immobilized urease can be used the determination of urea levels in provenders. Urease was isolated from *Cicer arietinum* and immobilization is performed in alginate beads for the first time and potential capacity of immobilized enzymes in the determination of urea in animal feed were investigated. Chickpea urease may be an important source for industrial and biotechnological applications.

Table 1. Provender Ingredients

Type of provenders	Analytical Components (%)	Additives (per kg)
A	16,0% crude protein, 11% crude cellulose, 2.6% crude oil, 8.5% crude ash, 0.3% ash, 2% urea	Vitamin A 5000 IU, vitamin D3 700 IU, mangane sulphate 78 mg/kg, iron sulphate monohydrate 211 mg/kg, zinc okside 48 mg/kg, copper sulphate pentahydrate 11 mg/kg, iodine 0.5 mg/kg.
B	14%crude protein, 12.1% crude cellulose, 4.3% crude oil, 0.43% sodium, 2% urea	Vitamin A 9600 IU, vitamin D3 1920 IU, iodine 0.8 mg/kg, Cobalt 0.15 mg/kg, copper 10 mg/kg, mangane 50 mg/kg, zinc 50 mg/kg, selenium 0.15 mg/kg
C	19% crude protein, 8.2% crude cellulose, 3.9% crude oil, 8.0% crude ash, 0.4% sodium	Vitamin A 12000 IU, vitamin D3 3000 IU, mangane sulphate 50 mg/kg, iron 123 mg/kg, zinc 53 mg/kg, copper 9 mg/kg, iodine 0.2 mg/kg
D	20% crude protein, 11.1% crude cellulose, 5.5% crude oil, 9.2% crude ash, 0.32% sodium	Vitamin A 10000 IU, vitamin D3 2000 IU, iodine 0.8 mg/kg, cobalt 0.15 mg/kg, copper 10 mg/kg, mangane 50 mg/kg, zinc 50 mg/kg, selenium 0.15 mg/kg

Table 2. Amount of urea in provender

Type of provender	Amount of urea (mg) *
A	8.05 (± 0.13)
B	7.95 (± 0.42)
C	8.70 (± 0.33)
D	9.11 (± 0.26)

*Measurements were obtained from the average of triplicate samples. Values in parentheses are standard deviations.

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