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Application of Immobilized α-Amylase onto Functionalized Calix[4]arene for Degradation of Starch

Irshad Ali Veesar[1]*, Shahabuddin Memon[1], Ranjhan Junejo[1], Imam Bakhsh Solangi[2]

[1] *National Centre of Excellence in Analytical Chemistry, University of Sindh, Jamshoro 76080, Pakistan*

[2] *Dr. M.A Kazi Institute of Chemistry, University of Sindh, Jamshoro 76080, Pakistan*

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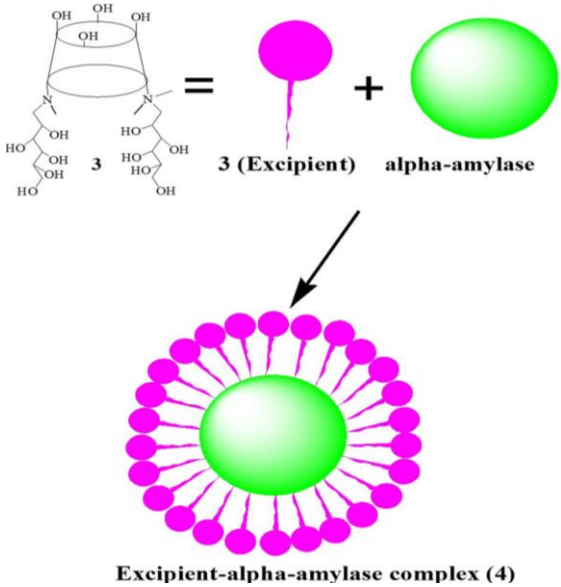
ABSTRACT

In the present study, water soluble 5,17-bis[(*N*-methylglucamine)methyl]-25,26,27,28-tetrahydroxycalix[4]arene (**3**, as a highly efficient excipient material) was synthesized to immobilize *α*-amylase first time using the *N*-methylglucamine functionalities for excipient-enzyme complex formation at its *para*-positions. The excipient-*α*-amylase complex (**4**) was used for the starch degradation. The optimum apparent activity of **4** was determined at variable conditions such as the effect of pH (7.0), temperature (25 °C) and initial concentration of enzyme (15 µL). Under such optimized parameters, the maximum 71% yield of enzyme was immobilized onto the functionalized calix[4]arene material (**3**). The catalytic properties of **4** were determined by comparing with free *α*-amylase. The complex **4** revealed high stability under sever conditions, i.e. high temperature and offers multiple reuses with little loss in enzyme activity due to higher α-amylase concentration has been protected by complex. In addition, the enzyme activity and the excipient-*α*-amylase complex were found to have extra characteristics as compared to the free α-amylase for starch hydrolysis with respect to its stability and reusability. These advantageous characteristics and low cost of material from which calixarene derivative was prepared, making it economically viable for starch degradation on industrial scale.

GRAPHICAL ABSTRACT



* Corresponding author: Veesar, Irshad Ali
✉ E-mail: irshadlatif82@gmail.com

Introduction

Enzymes are the important biocatalysts which are specific, highly active, and selective [1, 2]. These enzymes play major role in the field of biotechnology, food fermentation, pharmaceutical, paper, and textile industries [3-5]. Generally, amylase enzyme is applied for the degradation of starch amylo-pectin, glycogen, dextrin and other polysaccharides composed of many glucose units [6-8]. This kind of enzyme is widely present in animals, microbes and plants and have been used in several industries such as starch processing in food industry, laundry, decontamination of dyes from aqueous media starch liquefaction and production of ethanol and high glucose syrups are major applications [9, 10]. This amylolytic activity of starch degrading enzyme was explored in 1811 by Kirchhoff and α-amylase was proved to be a specific enzyme for this activity [11]. Although enzymes are more costly and difficult to isolate from natural sources, but their repetitive usage makes them inexpensive. However, the main difficulty associated with this phenomenon is their laborious and costly separation from the reaction mixture. Stabilization of the enzyme would lessen the need to buy fresh enzymes and hence reduces the cost of material production as a whole. One of the most effective ways to achieve stabilization is the use of immobilized enzymes [12].

The immobilized enzymes play a vital role in biotechnology, biomedical, analytical chemistry, and food technology [13-16]. Moreover, immobilized enzymes have achieved more importance as compared to free enzymes due to easy separation from reaction mixture and good % recovery after repeated usage [17, 18]. Therefore, different techniques have been applied for immobilization of enzymes such as copolymerization of enzymes, covalent attachment of enzymes to an activated support, glutaraldehyde cross linking of enzymes, physical adsorption or ionic interaction and their encapsulation onto polymeric matrices based solid supports [19]. The supporting polymer matrices should have high surface area as to immobilize maximum quantity of enzyme that may improve enzyme activity. On industrial scale, it is important that the supported material should have stable mechanical, chemical, thermal and physical properties. Thus, the material used as a fillers, binders and adhesives are known as excipient materials which have non-nutritional in food production but are very less/non-toxic in nature [20].

Nowadays, different enzyme support materials including, organic and inorganic, synthetic polymers and macromolecules have been extensively utilized [21]. According to immobilization protocols that support enzymes should have large surface area different materials such as zeolites, agarose and glass porous beads have been used previously [22]. Among them, the synthetic macromolecules such as calix[n]arenes are frequently applied due to high % yield and one pot synthesis using cheaper raw resources and have flexibility in their structure. Therefore, increasing the interest of these materials, have explored in drugs, chemical industries, nano fibers, and nano chips [22]. The calix[n]arene derivatives can be prepared and used as a better support for enzyme immunization [23]. Many studies have been conducted on evaluating the immobilization of enzymes onto calix[n]arene based materials such as calix[n]arene-based silica polymers were used as a solid support for *Candida rugosa* lipase enzyme [24]. Another study was performed using calix[4]arene derivative having the iminodicarboxylic/phosphonic groups and modified by iron oxide nanoparticles for the direct immobilization of *Candida rugosa* lipase enzyme through sol–gel encapsulation method [25]. In our previous studies, *Candida rugosa* lipase was immobilized onto sulphonate calix[4]arene [12] while α-amylase was

immobilized through covalent linkages between Ethylene diamine derivative of calix[4]arene [26].

In this research study, we have report the immobilization of α-amylase through excipient complex formation using *N*-methylglucamine calix[4]arene **(3)** to obtained better activity, thermally stable and reusable enzyme. Furthermore, the Calixarene is used as excipient material (inactive ingredient) and used as a carrier for the active ingredient (enzyme). During storage and manufacture or processing of the system; it protects, supports and enhances the stability as well as bioavailability of the bioactive compound.

Experimental

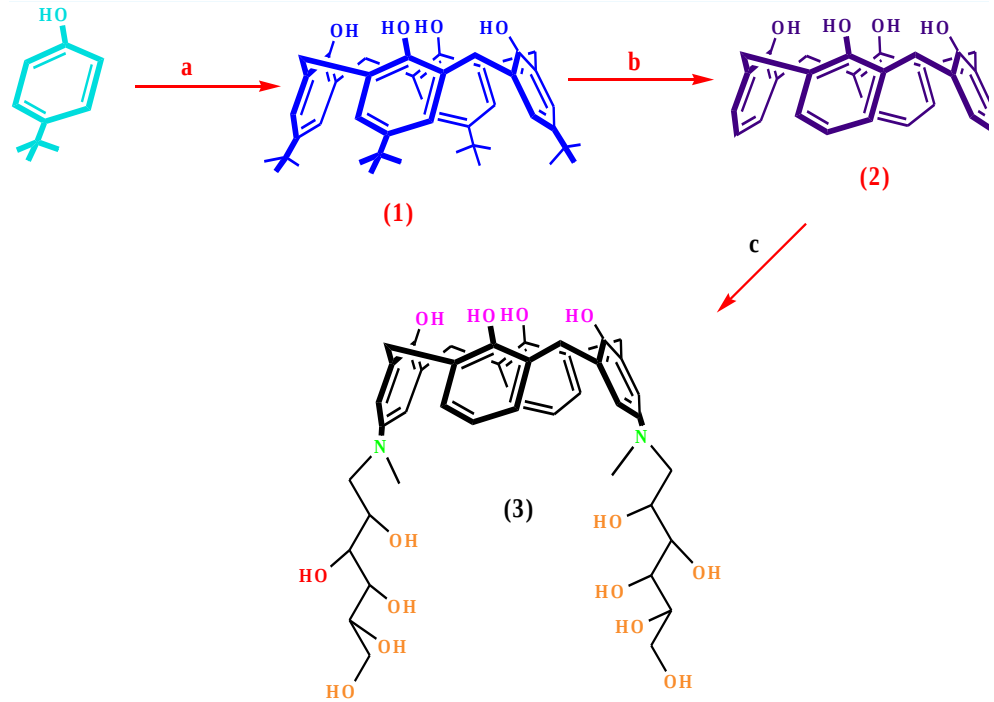
Materials and methods

The chemicals used in experimental study were analytical grades and were purchased from the Merck and Aldrich Company. The barley α-amylase, maltose, starch, bovine serum albumin (BSA), 3,5-di-nitrosalicylic acid (DNS), were procured from Sigma Aldrich.

All the aqueous solutions were prepared by deionized water. The enzyme activity was determined using UV–vis spectrophotometer (Agilent carry 100). The pH of solution was measured with pH meter (wtw) having glass electrode and internal reference electrode. A Centrifuge machine was used (*Andreas Hettich*) to carry out centrifugation of samples. Microstructure of the specimens was evaluated using a scanning electron microscope (SEM, JSM-5610LV; Jeol Ltd., Tokyo, Japan).

Synthesis

All the compounds (Scheme 1) were synthesized according to previously reported methods [27-30].



Scheme 1. Synthetic route for *N*-methylglucamine derivative of calix[4]arene (**3**) (**a**) $AlCl_3$/Phenol/Toluene; (**b**) fuming H_2SO_4; (**c**) *N*-methylglucamine, formaldehyde, CH_3COOH

Preparation of Excipient-α-Amylase Complex

Optimization of parameters for excipient-α-amylase complex formation

The excipient-α-amylase complex formation was determined by changing the concentration, incubation time, temperature and pH. During the experimental work, the substrate concentration (1% w/v) remains constant while all other parameters have been varied. The pH of solution was optimized using various buffers with the pH ranging from 4 to 9 (Table 1). To determine the maximum excipient-enzyme complex formation, the Bradford method was used [31]. The excipient (**3**) amount was adjusted using different concentrations of enzyme in shaker incubator for 1-6 h, and kept it for overnight at 25 °C.

Immobilization strategy

For the immobilization of α-amylase through complexation, 1 g of **3** were solubilized in 21 mL of phosphate buffer (0.02 M, pH 7.0), then 15 µL of (5 mg/mL) solution of enzyme was added and left for 5 h in water bath at 300 rpm and 25 °C. Immobilized enzyme was placed at 4 °C for 24 h and added n-Hexane to form precipitate of the immobilized enzyme. Finally the immobilized enzyme was separated from buffer and n-hexane solution through filtration techniques and confirmed by TLC with solvent system (acetone: n-hexane in 1:4 ratio) comparing the retardation factor (RF) value of compound **3** before and after immobilization. The results revealed that the compound **3** has RF value with respect to the immobilized-α-amylase. Keeping view of enzyme structure that calix[4]arene is a smaller than the therefore, in the proposed structure it is clear that many calix[4]arene molecules will immobilize onto a single molecule of an enzyme, as shown in Figure 1. Here in this research study, the enzyme is immobilized through excipient-complex formation, where calix[4]arene derivative is used as excipient material, which forms complex with the enzyme in buffer (pH=7, 0.02 M) through *N*-glucoamine functionalities. Calixarene molecules act as protectors/supports of the system during preparation of the complex and enhance the stability, bioavailability of the bioactive compound during storage or usage. The enzyme is immobilized through electrostatic interactions between enzyme and binding sites of calixarene that result in complex formation. In the enzyme complex formation, the unbound enzymes have washed with the buffer solution. With the help of these washings the amount free released enzyme can be calculated. The Bradford (1976) method was applied using Coomassie Brillant Blue reagent was to measure enzyme protein concentration in extract [31]. All the experimental results were repeated three times in order to measure the average excipient-α-amylase complex formation using the Equation (1).

$$\%\ Excipient - Amylase\ complex\ formation = \frac{Specific\ Activity\ of\ EAC}{Specific\ Activity\ of\ Free\ Amylase} \times 100 \quad (1)$$

Evaluation of free and complex α-amylase activity

The enzymatic activity of the free and complexed α-amylase have been examined using DNS procedure [32]. The 1 unit of an enzyme activity is considered as the amount of enzyme required to produce 1 μmol of reducing sugar/min. In this study, the excipient-amylase complex activity was determined using 1 mL of starch solution (1%) diluted up to 10 mL with the addition of phosphate buffer (0.02 M, pH 7), and 1 g of excipient-enzyme complex was mixed and placed in an incubated shaker at 25 °C for 10 min. The reaction was stopped by addition 1 mL of 5% DNS solution and 1 mL of this mixture was drawn off and heated for 15 min, and then diluted with 5 mL water. The sample was analyzed through UV-visible spectrophotometer at the λmax 540 nm. Appropriate blanks were prepared without α-amylase. For free α-amylase activity determination 1% starch solution was used and 0.1 mL (5 mg/mL) of enzyme solution

was used, further procedure was followed as described as above.

Storage stability test

The storage stability of the excipient-α-amylase complex was checked for 55 days by examining the activity on every day and then placed it in refrigerator.

Results and Discussion

For enzyme immobilization, the temperature stability and cost of support material is very crucial criteria. Herein, α-amylase has been immobilized through excipient-α-amylase complex formation with compound **3**, which is cheaper, stable, and reusable material. Moreover, for the complex formation there is no any need of any chemical modification of compound **3** because it has high solubility in water and can easily form complex with α-amylase enzyme in single step. Figure 1 demonstrates the proposed mechanism of complex formation. This complex formation approach may not only enhance the activity of that enzyme but also it may increase its thermal stability and provides a potential bio catalytic material which have high resistant against physical and chemical changes.

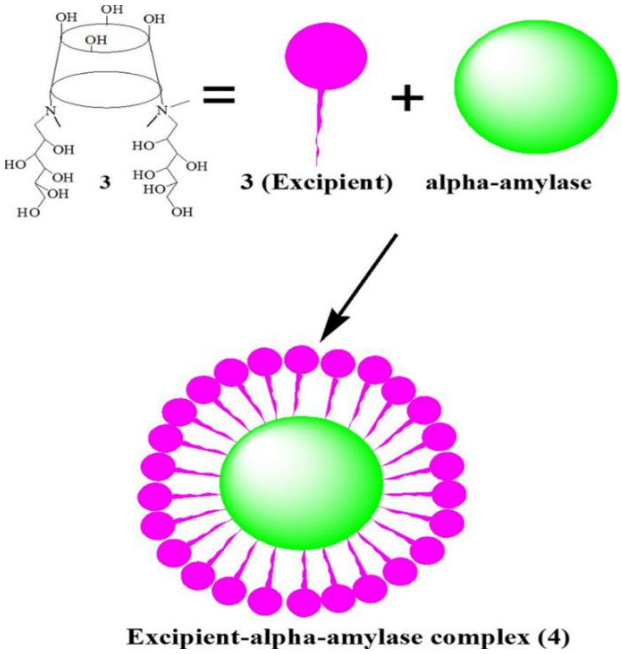


Figure 1. Proposed schematic representation of excipient-α-amylase complex (**4**) formation

The effect of conditions on excipient-α-amylase complex formation

During the excipient-α-amylase complex formation, different parameters such as concentration, time, pH and temperature effects have been optimized as listed in (Table 1).

Table 1 shows that 1 g of excipient with 15 µL enzyme at pH 7 and 25 °C temperature gives maximum (71%) yield. The other higher and lower concentrations were less significant and have less efficiency.

Microstructure analysis

Morphology of **3** and the excipient-α-amylase complex (**4**) were examined using the scanning electron microscope (SEM). Figure 2a demonstrates the SEM images of **3** which are clear uniform and smooth while Figure 2b is the image excipient-α-amylase complex (**4**) formed after the coatings of **3** are rough and amorphous.

Figure 2. SEM images of (a) Excipient (**3**) and (b) Excipient- α-amylase complex (**4**)

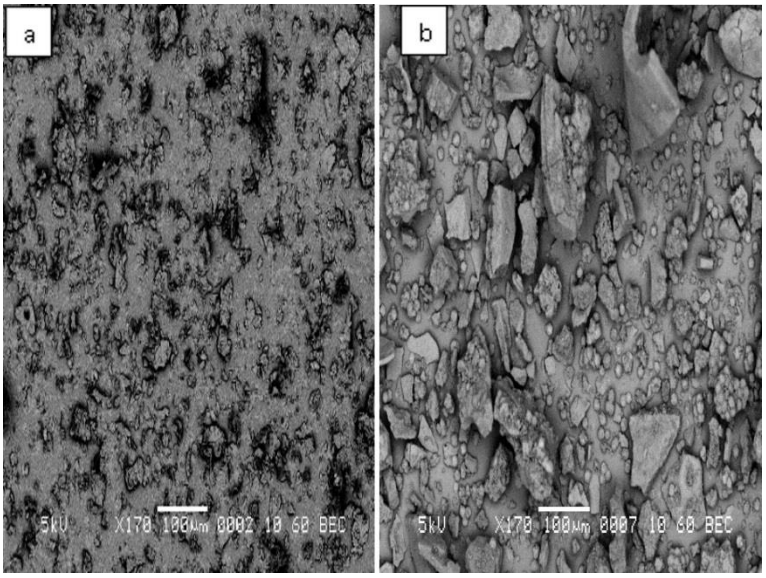


Table 1. Optimized conditions for excipient-α-amylase complex formation

Conditions	Enzyme loaded (µL)	Incubation (h)	pH	Temperature (°C)	Excipient- *α*-amylase Complex (%)
Enzyme loaded (µL)	5	3	7	25	46
	10	3	7	25	57
	15	3	7	25	71
	20	3	7	25	69
	25	3	7	25	55
Incubation time (h)	20	1	7	25	33
	20	2	7	25	48
	20	3	7	25	69
	20	4	7	25	66
	20	5	7	25	54
pH	20	3	4	25	38
	20	3	5	25	49
	20	3	6	25	67
	20	3	7	25	78
	20	3	8	25	66
	20	3	9	25	54
Temperature (°C)	20	3	7	20	58
	20	3	7	25	72
	20	3	7	30	70
	20	3	7	35	66
	20	3	7	40	52
	20	3	7	45	41
Excipient (g)	0.25	3	7	25	31
	0.5	3	7	25	43
	0.75	3	7	25	57
	1.0	3	7	25	76
	1.25	3	7	25	72
	1.5	3	7	25	69

Effects on Enzyme Activity

Effect of pH

The enzyme activity depends on pH conditions and show diverse activity at varying pH values. The pH of free and the immobilized α-amylase enzymes were examined during the hydrolysis of starch. The experiments were conducted at the pH range of 5-9 at 25 °C to determine the activity of excipient-α-amylase complex **4**. The complex showed maximum activity at pH 7.0, as seen in Figure 3.

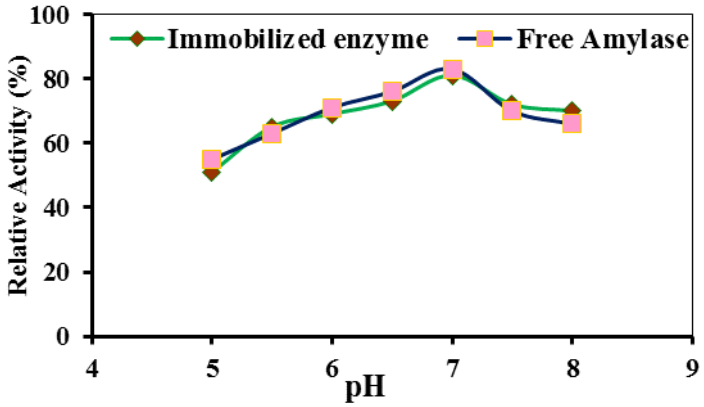


Figure 3. Effect of pH on the activity of free and complexed α-amylase (**4**)

Effect of temperature

The fundamental aim of this study was to increase the temperature stability of excipient-α-amylase complex **4.** So different temperatures (25-95 °C) were studied to evaluate the activity of free and the complexed α-amylase (Figure 4). The results showed that the maximum thermal stability of free α-amylase is 35 °C while the complex **4** has 70 °C. At higher temperature the stability of the free enzyme was decreased readily as compared to complex **4**, thus, the temperature stability complex **4** was much better as compared to free enzyme.

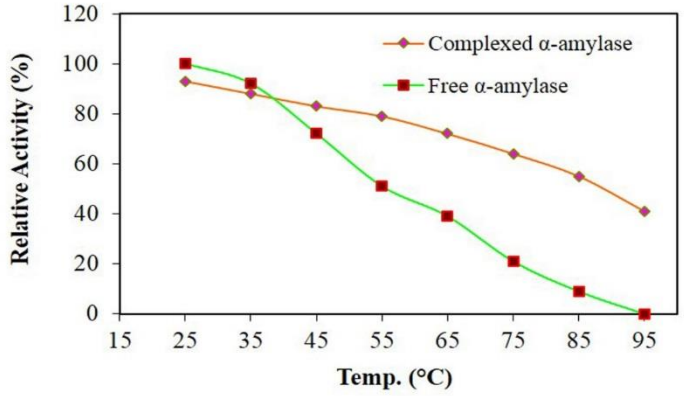


Figure 4. Effect of temperature on the activity of free and complexed α-amylase (4)

Reusability of the excipient-α-amylase complex 4

The reusability of an enzyme is a very important factor, affecting its utilization on industrial and commercial scale. Therefore, the re-usage study of excipient-α-amylase complex **4** was performed for the production of maltose from starch. During the experimental study, 15 re-usage cycles were evaluated shown in Figure 5. The results demonstrated that the enzymatic activity was maximum up to six cycles and decreased up to 50% after 10 cycles. The little loss of enzymatic activity after each cycle is due

to denaturing of enzyme as well as physical loss of enzyme from the carrier matrices.

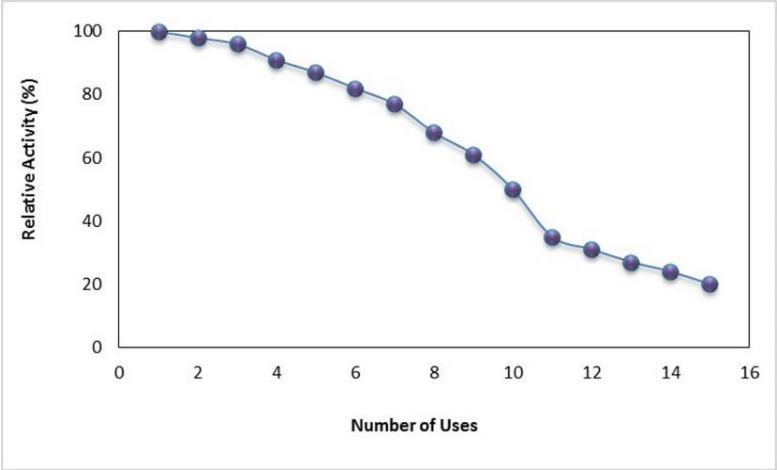


Figure 5. Reusability study of complexed α-amylase (**4**)

Conclusion

In this research study, a green and cheaper pathway for *α*-amylase immobilization in the form complex **4** was prepared. The α-amylase was stabilized through *N*-glucoamine functionalities of compound **3** by complex formation at the presence of phosphate buffer. Moreover, to assess the stability and activity of the free α-amylase and complex **4**, different studies were performed and it is observed that the complex **4** has high stability and activity as compared to free α-amylase. Moreover, the complex 4 revealed better reusability for their utilization on commercial and industrial scale.

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Disclosure statement

No potential conflict of interest was reported by the authors.

ORCID

Ranjhan Junejo : 0000-0002-0090-1146

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