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Immobilization of amylase onto arylamine glass beads affixed inside a plastic beaker: Kinetic properties and application

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Commercial fungal amylase (diastase) has been immobilized through diazotization onto zirconia coated arylamine glass beads, affixed inside a plastic beaker. The immobilized enzyme retained 73.3% of the initial activity of free enzyme with a conjugation yield of 8 mg/g. Optimum *p*H of enzyme was decreased, while temperatures for maximum activity, energy of activation, time of incubation and K_m for starch were increased after immobilization. The utility of immobilized enzymes in removal of starch stain from cotton cloth by various detergents was tested by chemical method. All the detergents gave better washing in presence of immobilized amylase than that by detergent alone. Further, the washing by cheaper (non-enzymic) detergents in presence of immobilized amylase was almost similar to that by expensive (enzymic) detergents. The immobilized enzyme was used about 100 times without any considerable loss of activity.

Keywords: amylase, arylamine glass, cloth washing, detergent, immobilization, starch stain.
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Introduction

Immobilization of commercially available fungal amylase (diastase) onto affixed alkylamine glass beads through glutaraldehyde coupling and its usefulness in removal of starch stain from cotton cloths has been demonstrated in this laboratory[1]. Though a better washing of cotton clothes was found in presence of alkylamine glass bound amylase compared to the detergent alone, immobilization of enzyme onto alkylamine glass beads through glutaraldehyde involves Schiff's base formation has the drawback of reversibility of the enzyme reaction. However, immobilization of enzyme onto arylamine glass beads through diazotization has no such problem. Hence the present report.

Materials and Methods

Chemicals

Zirconia coated arylamine glass beads (pore diameter 55 nm) were gift from Prof. H H Weetall, Environment Protection Group, Las Vegas, U.S.A.; α-amylase, sodium potassium tartarate, dinitro salicyclic acid (DNS), anthrone and starch were from SISCO Research Laboratories Pvt Ltd., Mumbai and detergents were purchased from local market. All other chemicals used were of AR grade.

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Assay of α-Amylase

Amylase assay was carried out as described by Mifflin and Bruns[2] with modification. It was based on measurement of glucose and maltose generated from hydrolysis of starch by α-amylase using DNS reagent. Unit of amylase is defined as the amount of enzyme required to liberate 1 μmole of glucose from starch per min under the standard assay conditions (at 37°C, *p*H 5.6).

Affixation of Arylamine Glass Beads

The inner side of a 100 mL plastic beaker was scratched with a sand paper and then coated uniformly up to 1 cm height with the fixative 'Araldite'. About 100 mg powder of arylamine glass were sprinkled uniformly on this fixative layer and allowed to stand at room temperature for 24 h.

Immobilization of Amylase onto Affixed Arylamine Glass Beads

Immobilization of amylase was carried out as described by Lynn[3]. Arylamine glass beads affixed inside a plastic beaker (100 mg) were diazotized, kept in an ice bath and 100 mg solid $NaNO_2$ and 2 mL 2 *N* HCl were added to the beaker and kept for 30 min in ice bath. After that excess of HCl was decanted and diazotized glass beads were washed many times with 0.1 *M* sodium phosphate buffer (*p*H-7.0) until the *p*H of discard was 7.0. Amylase solution (2 mL) was added to the activated beads and then left for 48 h at

4-10ºC with gentle shaking at an interval of 2 h. The unbound enzyme was decanted and tested for activity and protein[4]. The glass beads were washed 3-4 times with the same buffer, until no activity of enzyme was detected in the washing. The enzyme protein bound to glass beads was estimated by determining the loss of protein from the solution during immobilization[4].

Assay of Immobilized Enzyme

To 1.9 mL 0.05 *M* acetate buffer (*p*H 5.6) containing 2% starch in a test tube, 0.1 mL of enzyme solution was added. For blank, 2 mL of buffer containing starch was taken in a test tube. Both tubes were incubated at 37°C under continuous stirring for 10 min in a water bath. After which 0.1 mL 2 *N* NaOH and 0.9 mL DNS reagent was added to both the test tubes. The tubes were placed in a boiling water bath for 5 min, cooled to room temperature and A_{540} of red coloured mixture was read. The amount of glucose generated in the reaction mixture was extrapolated from standard curve between glucose concentration and A_{540}[2].

Application of Immobilized Amylase

The immobilized amylase was used for removal of starch stain from cotton cloth in the presence of solution of commercial detergents. For this purpose, the cotton cloths pieces (size: 4.5×4.5 cm^2) were stained with 0.2 mL of aqueous starch solution. The stock solutions of eight commercially available detergents, namely Surf Excel, Ariel Compact, Rin Supreme, Multi-action Surf, Henko, Rin Shakti, Nirma and Wheel, in distilled water (2.0 g/L) were prepared. Two stained cloth pieces were taken for each detergent. One piece was washed with detergent alone, while another piece was washed with detergent in the presence of immobilized amylase at 45°C for 20 min with continuous stirring. Similarly, one stained piece was washed in distilled water and immobilized amylase. Each washed cloth piece was rinsed twice manually with distilled water. The washing performance in each was determined as follows[1]. The cloth was treated with 5 mL hot water, then squeezed and supernatant was collected. This was repeated 3 times. All the fractions were combined and the volume was made up to 100 mL with distilled water. 5 mL of it was taken in a tube and 10 mL freshly prepared anthrone reagent was added (anthrone reagent consisted of 2 gm solid anthrone dissolved in 100 mL of cold 95% conc. H_2SO_4). Tube was placed in boiling water bath for 10 min and cooled at room temperature. Then absorbance at 630 nm was read.

Results and Discussion

Commercially available fungal amylase has been immobilized onto aryl amine glass beads through diazotization with a conjugation yield of 8 mg/g support. The immobilized enzyme retained about 73.3% of the initial specific activity of the soluble/free enzyme.

Kinetic Properties of Immobilized Amylase

A comparison of kinetic properties of amylase coupled to arylamine glass beads with those of free enzyme and affixed alkylamine glass beads is given in Table 1. The maximum activity of arylamine glass beads bound amylase was attained at *p*H 5.5, which is lower than that of free enzyme (*p*H 5.6)[1]. Optimum *p*H of an enzyme is displaced upon immobilization, particularly when the support material is charged. The immobilized amylase showed maximum activity at 45°C, which is higher of free enzyme (37°C)[1]. The energy of activation (E_a) of the immobilized enzyme was increased from 4.43 Kcal/mol to 5.34 Kcal/mole after immobilization. The time of incubation was also increased from 10 to 20 min after immobilization, which might be due to diffusion of the substrate from bulk to the active center of the immobilized enzyme. The substrate (starch) concentration required for the maximum activity or saturation of immobilized amylase was two times lower than that of free enzyme. K_m for starch, as calculated from Lineweaver-Burk plot, was increased as compared to free enzyme (13), indicating the decreased affinity of the enzyme towards the substrate (starch) after immobilization. V_{max} was changed from 0.06 to 0.02 µmol/min after immobilization. The changes in kinetic properties of enzyme after immobilization are

Table 1—A comparison of kinetic parameters of free amylase and enzyme bound to affixed arylamine glass beads

Parameter	Free Amylase[1]	Amylase conjugated to affixed arylamine glass beads (present)
Optimum *p*H	5.6	5.5
Temperature for maximum activity	37°C	45°C
E_a (Kcal/mol)	4.43	5.34
Time of incubation (min)	10	20
K_m for starch (m*M*)	15	8
V_{max} (n*M*/min)	0.06	0.02
Storage stability in (D.W. at 4°C)	--	2 months

controlled by four factors: change in enzyme conformation and its microenvironment, steric effects, and bulk and diffusional effect[5].

Application of Immobilized Amylase

The starch stained cotton cloth pieces were washed with detergents alone and in presence of immobilized amylase. The residual starch in cloth pieces was determined. A standard curve was prepared by taking varying concentration of glucose in the range 0-100 μg. The value of glucose was multiplied by 0.9 for conversion of glucose. Minimum amount of starch content indicates better washing. Two types of detergents were tested, expensive (enzymic) detergents such as Surf Excel, Ariel Compact, Multi-action Surf, Henko and non-enzymic detergents such as Rin Shakti, Nirma, Rin Supreme and Wheel. The washing performance of the detergents was in the following order: Surf Excel>Ariel Compact>Rin Shakti>Multi-action Surf>Rin Supreme>Henko>Nirma>Wheel> distilled water (Table 2). However, in distilled water, all detergents gave similar washings in presence of immobilized amylase. The combination of any detergent + immobilized amylase gave better washing than that by detergent alone, because the residual starch content was found less in case of detergent + immobilized enzyme. Amylases are known to hydrolyze the starch and, thus, remove the starch stain from the clothes freely and rapidly.

Detergents normally contains surfactants, builders, co-builders, bleach, bleach activators and special additives, such as fluorescent brightener, filler, corrosive inhibitors , antifoaming agents and enzymes (in case of only enzymic detergents) and perfumes. Surfactants, a major components of detergents are of four types: (i) anionic (e.g. sodium lauryl sulfate), (ii) cationic (e.g. hexadecyltrimethylammonium bromide as fabric softener), (iii) non-ionic (e.g. *n*-odecyloctaethylene glycomonoether ethoxylate), and (iv) amphoteric (e.g. laurylamido propyl dimethyl betaine as skin cleaner). A detergent may contain more than one type of surfactant. Hence, it is not possible to know the overall ionic state of a detergent, especially when the chemical composition of a commercial detergent is not available, due to professional secrecy. In the present case, the enzyme (α-amylase) is bound to arylamine glass beads through covalent bonding. These glass beads are affixed on inner wall of plastic beaker. The enzyme in this form is hardly affected by the surfactant in solution. Hence, the comparison of washing performance of various detergents in the presence of various immobilized enzyme is due to their individual performance but not due to their different effect on the enzyme. The immobilization of enzyme onto inert surface protects it from the effect of surfactant as well as provides its reuse[6]. The immobilized amylase was reused about 100 times in such washings without any considerable loss of activity. Generally amylase in free from is not safe as this might be attacked by proteases and inhibited by surfactant. Thus, the use of arylamine glass beads bound amylase in washing of starch stained cloths by detergents has not only increased their washing efficiencies without consuming them in the process but also made cheaper detergents equivalent to expansive detergents for washing purpose.

Table 2—Colorimetric determination of residual starch content in the cotton cloth after washing with various detergents alone and in the presence of immobilized amylase

Detergent used	Starch content mg/mL	
	Detergent alone	Detergent + immobilized amylase
Surf Excel	0.490	0.365
Ariel Compact	0.870	0.780
Henko	1.000	0.955
Multi Action Surf	0.900	0.825
Rin Shakti	1.000	0.920
Nirma	1.250	1.230
Rin Supreme	1.600	0.537
Wheel	1.200	1.187

A standard curve was prepared by taking varying concentration of glucose in range 0-100 μg glucose. The value of glucose was multiplied by 0.9 for conversion of glucose value to starch. Minimum amount of starch content indicates better washing. The washing performance of the detergents was in the following order: Surf Excel>Ariel Compact>Rin Shakti>Multi-action Surf>Rin Supreme>Henko>Nirma>Wheel>distilled water. In distilled water all detergents gave similar washing in presence of immobilized amylase.

Storage and Stability

The immobilized amylase on arylamine glass beads showed no noticeable loss of activity during its regular uses (100 times) for about 2 months, when stored in distilled water at 4°C. The half life ($t_{½}$) of immobilized enzyme was 3 months.

Conclusion

Commercially available fungal amylase has been immobilized onto affixed arylamine glass beads. Some changes in the kinetic properties of enzyme were observed after immobilization. The immobilized enzyme was employed in removal of starch stain from

cotton clothes by various detergents. The washing by cheaper (non-enzymic) detergents in presence of immobilized enzyme was almost similar to that by expensive (enzymic) detergents. The immobilized enzyme was reused about 100 times without any loss of its activity.

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