WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 5.045
Volume 4, Issue 1, 1218-1230. Research Article ISSN 2277– 7105

APPLICATIVE BIODEGRADATION STUDY OF EGG ALBUMIN NANOSPHERES BY ALKALINE PROTEASE FOR RELEASE OF ENCAPSULATED *CICER ARIETINUM* AMYLASE IN WASHING AS BIO-ACTIVE DETERGENT ADDITIVE

Kirti Rani*

Amity Institute of Biotechnology, Amity University Uttar Pradesh, Noida,, Sec-125, Noida-201303 (UP), India.

Article Received on
02 Nov 2014,

Revised on 27 Nov 2014,
Accepted on 22 Dec 2014

***Correspondence for Author**
Dr. Kirti Rani
Amity Institute of Biotechnology, Amity University Uttar Pradesh, Noida, Sec-125, Noida-201303 (UP), India.

ABSTRACT

In present work, *Cicer arietinum* amylase was encapsulated through covalent coupling by glutaraldehyde into chemically modified egg albumin nanospheres using toluene as stabilizer. Biodegradation of chemically modified egg albumin nanospheres was carried out by using varying concentration of alkaline protease (5U, 10U, 15U, 20U, 25U, 30U, 35U, 40U) for its controlled and sustained release of encapsulated enzyme. Storage stability and thermal stability of encapsulated enzyme was increased up to 6 months and up to 70ºC respectively as compared to free enzyme (24 hours only and 50ºC respectively) which enhanced industrial application of the bio-active enzyme loaded egg albumin nanopreparation. Further, chemically modified egg albumin nanospheres of encapsulated *Cicer arietinum* amylase with alkaline protease were used with detergents for washing of stained cloths having tough dried strains of ball point pen, white board marker and permanent marker and not to be easily vanished with detergents in one wash. Hence, the bio-active mixture of *Cicer arietinum* amylase loaded chemically modified egg albumin nanospheres with alkaline protease were used with detergents powder (Aerial, Surf excel, Wheel and Tide) for washing of these dry tough strains (ball point pen, white board marker and permanent marker) lead to washing off strains very fast with clear results in one wash without soaking as compared to results of washing of stained cloths with detergents only.

KEYWORDS: *Cicer arietinum* amylase, egg albumin, Glutaraldehyde, Encapsulated, Emulsified.

INTRODUCTION

Amylases catalyze the hydrolysis of glucosidal linkages in starch polymers and have wide applications in a number of industrial processes such as food, fermentation, textile, paper, detergent and pharmaceutical industries.[1,2,3,4] Amylases are also used in gelatinization (100-110°C) and liquefaction (80-90°C) as thermostable enzymes used in starch industry.[5,6] After immobilization, amylase has increased storage stability, thermal stability because it prevents excessive loss of enzyme activity and protects enzyme from microbial contamination. Nanoparticles are stable, solid colloidal particles made up of biodegradable polymers and are being used for drug delivery because of their lower toxicity, increases drug solubility, increases bioavailability and target drug delivery as better intravenous delivery system.[7,8] Further, albumin being a non-toxic, biodegradable, biocompatible protein carrier in drug delivery is shown to be an ideal matrix to fabricate enzyme or drug loaded nanoparticles for drug delivery.[9,10] Moreover, albumin nanoparticles can be easily prepared by emulsion formation, coacervation or controlled desolvation and commercially, albumins are obtained with significant quantities from egg, bovine serum albumin, human serum albumin and also available from soybeans, milk, and grains.[11] Previously, egg albumin nanoparticles with size 100nm had been prepared by Iranian scientists by simple coacervation method for drug delivery systems.[12] In our work, egg albumin was used as cheap eco-friendly biomatrix for encapsulation of *Cicer arietinum* amylase which was chemically modified by butanol and stabilized using toluene, coconut oil and glutaraldehyde. The enzyme loaded egg albumin nanospheres preparation involved the formation of a water-in-oil emulsion and subsequent stabilization of the protein droplets by using glutaraldehyde as a cross-linking agent.[13,14,15] The characterization of enzyme loaded egg albumin nanospheres was done by using Dynamic Light Scattering (DLS) and Scanning Electron Microscope (SEM) techniques.[16] Biodegradation of egg albumin nanospheres was done by using varying units of alkaline protease (5U, 10U, 15U, 20U, 25U, 30U, 35U, 40U) for release of encapsulated enzyme and subjected to washing of the stained clothes with detergents.[13,15,17,18] The applications of chemically modified egg albumin nanospheres of encapsulated *Cicer arietinum* amylase was studied with chosen samples of washing powders (Ariel, Surf excel, Wheel and Tide) for washing of dry tough stained clothes pieces (ball point pen, white board marker and permanent marker).[11,18]

MATERIALS AND METHODS

Extraction of amylase enzyme from *Cicer arietinum* seeds

Cicer arietinum seeds were soaked for 3days to get 3-days seedlings, crushed in the pestle mortar and homogenized by adding 4-6 ml of 0.05 M sodium phosphate buffer (pH 7.0) per gram of seedlings. It was centrifuged for 15mins at 4° C at 5000rpm. Supernatant was collected which contained crude amylase extract and stored at 4° C.[15]

Amylase Assay

Enzyme assay was done by using 1 %(w/v) starch solution in which 0.5 ml enzyme extract was added. It was incubated at 37°C for 20 minutes. 2 ml of dinitrosalicylic acid was added and the mixture was boiled at 100°C for 5 minutes. Absorbance was taken at 570nm.[15, 19-20]

Purification by Ammonium Sulphate Precipitation and Dialysis

80% of ammonium sulphate precipitation was done in ice bath by adding ammonium sulphate salt in to crude enzyme extract and stirred with glass rod for 25 minutes after each and every addition of salt. After the complete addition of ammonium sulphate salt was stirred for 30 minutes and incubated for 1 hour and centrifuged for 25 minutes at 4°C at 10000rpm. Supernatant was discarded and the pellet was dissolved in 0.05 M sodium acetate buffer (pH 3) and kept at -20°C. Dialysis was done in partially purified enzyme extract by using dialysis tubing which was rinsed with distilled water whose one end was clutched with a thread and 10 ml of sample was poured into it and other end was also clutched called dialysis bag which was placed in 10mM Tris HCl buffer. It was incubated for 24 hours at 4°C. The pure sample was poured out after incubation and kept at 4°C.[21-22]

Encapsulation of *Cicer arietinum* Amylase into Chemically Modified Egg Albumin

Oil bath was prepared with a solution of 25% glutaraldehyde, 2.6 ml of n-butanol, 4-5ml of toluene and 50ml of coconut oil. 50U *Cicer arietinum* amylase was added in 8-10 ml of egg albumin was taken in a 10 gauge syringe. It was dispersed in prepared oil bath and kept overnight in incubator shaker at 37°C. Next day, it was centrifuged at 5000rpm at 4°C for 20 minutes. Supernatant was removed. Pellet was washed with cold diethyl ether and acetone.[23] Diethyl ether was added to the washed pellet and sonicated to get fine sized enzyme loaded egg albumin nanospheres. Enzyme assay was done in supernatant to know the % of encapsulation of enzyme in chemically modified egg albumin nanopsheres.[16,17, 24]

% of Encapsulation

The % of encapsulated enzyme was calculated by determining the residual enzyme activity from reaction mixture in which encapsulation of enzyme was done. Amylase assay was performed by using dinitrosalicylic acid method. [16,17]

$$\% \text{ of encapsulated enzyme} = \frac{\text{Specific activity of encapsulated enzyme}}{\text{Specific activity of free enzyme}} \times 100$$

Study of Kinetic Properties

The free enzyme and immobilized enzyme are characterized for their different kinetic properties i.e. effect of pH, effect of temperature, effect of incubation time, effect of CaCl2 concentration and effect of substrate concentration.[15,21,22] The effect of pH on activity of free and encapsulated enzyme was studied by performing enzyme assay at varying pH (2 to 12). The effect of time on the activity of free and encapsulated enzyme was studied by performing the enzyme assay at different time (5 minutes-25 minutes). Optimal substrate concentration needed for maximal enzyme activity for free and encapsulated enzyme which were estimated by incubating the reaction mixture at different concentrations of starch solution (0.25%-1.50%). The effect of $CaCl_2$ on activity for free and encapsulated enzymes was studied by performing the enzyme assay at different concentrations (2%-10%). Optimal temperature needed for free and encapsulated enzyme for maximal activity was studied by incubating the reaction mixture for 15 minutes at different temperature (10°C – 100°C). These kinetic properties of enzyme were determined by performing dinitrosalicyclic acid method.[15,19,20]

Biodegradation Study of Chemically Modified Egg Albumin Nanospheres by Alkaline Protease for Controlled Release of Encapsulated *Cicer Arietinum* Amylase

2 mg of enzyme loaded chemically modified egg albumin nanopsheres was taken in test tubes with reaction solution of different units of alkaline protease (5U, 10U, 15U, 20U, 25U, 30U, 35U, 40U) from 1st day till 8th consecutive days. The reaction tubes were incubated at 4°C for overnight. Next day, enzyme assay was done at 570 nm using dinitrosalicylic acid method.[19-22, 25-26]

To Study Washing Application of Chemically Modified Egg Albumin Nanospheres of Encapsulated *Cicer Arietinum* Amylase in Desizing of Stained Cloths

Chemically modified egg albumin nanosphers of encapsulated *Cicer arietinum* amylase were used in vanishing of dry tough stained cloths (Ball point pen, white board marker and permanent marker) in combination of various detergents such as Ariel, Tide, Surf and Wheel. Different stained cloth pieces (Ball point pen, white board marker and permanent marker) were chosen and washed in reaction solution of 2 mg of prepared enzyme loaded egg albumin nanospheres with 1ml of detergent solution. Each sample of stained cloths pieces was tested with all the four detergents (Aerial, Surf excel, Wheel and Tide) only and with the combination mixture of enzyme loaded chemically modified egg albumin nanospheres for carrying out its comparative washing study.[18, 25]

RESULTS AND DISCUSSION

% of Encapsulation

Chemically modified egg albumin nanospheres of encapsulated *Cicer arietinum* amylase had 80% retention of enzyme activity whose result was similar to previous study in which *Glycine max* amylase was bound into chemically modified bovine serum albumin with 85% retention of activity.[14, 16]

Studied Kinetic Properties

The pH was varied from 2 to 12. In present study, the result obtained for optimal pH of encapsulated *Cicer arietinum* amylase had maximum activity at pH 6.0 which was lower than the free enzyme 8.0 whose results were comparable to previously studies.[15,21,22] The optimal incubation time was found for encapsulated enzyme was 25 minutes which is higher than the free enzyme (15 minutes) which are pretty similar to previous finding.[15,21] Optimal substrate concentrations of encapsulated enzyme was found 1.25% which is higher than the free enzyme 1% which are also pretty comparable to past finding.[15,20,21,22] Optimal $CaCl_2$ concentrations was found 4% for encapsulated enzyme and 8% for free enzyme whose results was similar to previous results.[15,21] Optimal temperature was found 60°C for encapsulated enzyme as compared to free enzyme which was 50°C. It was found that after encapsulation, thermal stability was increased as compared to free enzyme which is also sharply comparable to previous finding (Table 1).[15,20,21,22]

Table 1. Studied Kinetic Parameters of free and encapsulated *Cicer arietinum* amylase.

Kinetic Parameters	Free amylase	Encapsulated amylase
pH optima	8.0	6.0
Optimal time of incubation	15 minutes	25 minutes
Optimal Substrate concentration	1.00%	1.25%
Optimal $CaCl_2$ concentration	8%	4%
Optimal Temperature	60ºC	50ºC
Thermal Stability	Up to 50ºC	Up to 70ºC
Storage Stability at 4ºC	Up to 24 hours	Up to 6 months

Characterization of *Cicer Arietinum* Amylase Loaded Chemically Modified Egg Albumin Nanospheres

Prepared *Cicer arietinum* loaded chemically modified egg albumin nanospheres was showed the presence of nanospheres when subjected to Dynamic Light Spectroscopy (DLS) (Fig 1). The sample was also observed under Scanning Electron Microscope (SEM) showed spherical particle with size ranging from 101.2nm to 157.4nm which were pretty comparable to lesser than the previous studies (Fig 2).[16]

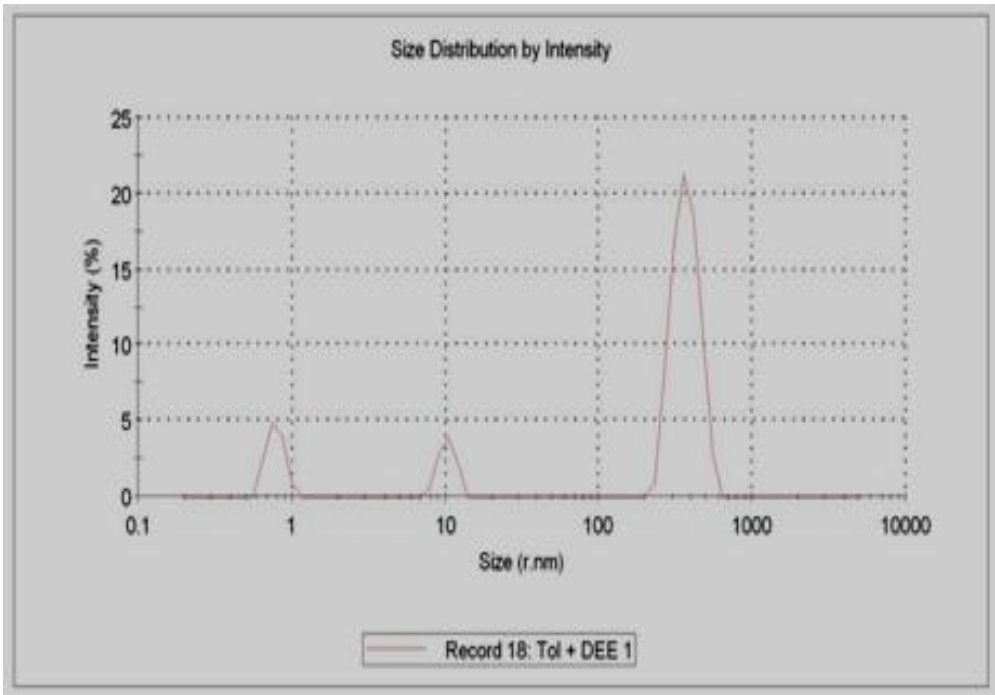

Fig. 1: DLS of chemically modified egg albumin nanospheres of encapsulated *Cicer arietinum* amylase.

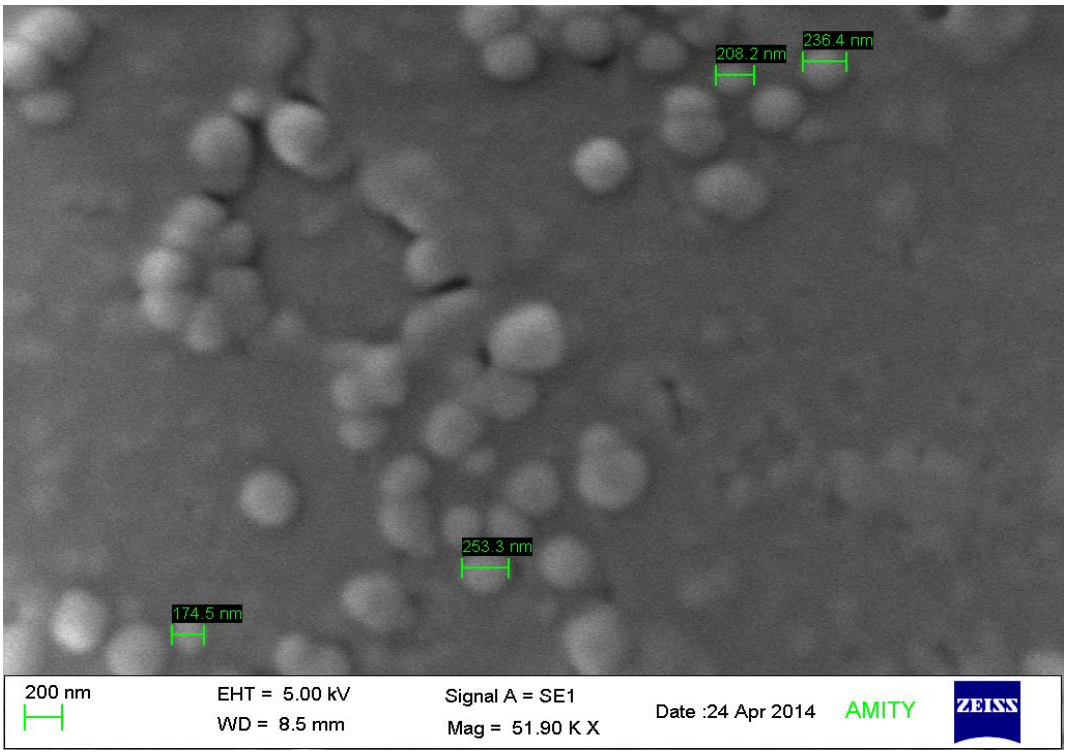

Fig. 2: SEM of chemically modified egg albumin nanospheres of encapsulated *Cicer arietinum* amylase.

Biodegradation Study

Biodegradation study was performed by incubating 2 mg of chemically modified egg albumin nanospheres of encapsulated *Cicer arietinum* amylase with varying units of alkaline protease (5U, 10U, 15U, 20U, 25U, 30U, 35U, 40U) overnight at 4°C. The study was carried out for consecutive 8 days. It was found that 35U of alkaline protease was effective to achieve controlled and sustained release of encapsulated *Cicer arietinum* amylase form chemically modified egg albumin nanospheres (Fig 3). Alkaline protease was already coined the best suited industrially important thermostable enzyme to adjust with pH of washing powder.[28] Biodegradation pattern for each incubating mixture was showed an increase from 1st to 3rd day, decrease on 4th day and remain constant till 6th day whose results were pretty much similar to the previous study in which entrapped *Vigna radiata* amlyase, was released from chemically modified bovine serum albumin with 30U of alkaline protease solution.[14, 16,17, 22, 29]

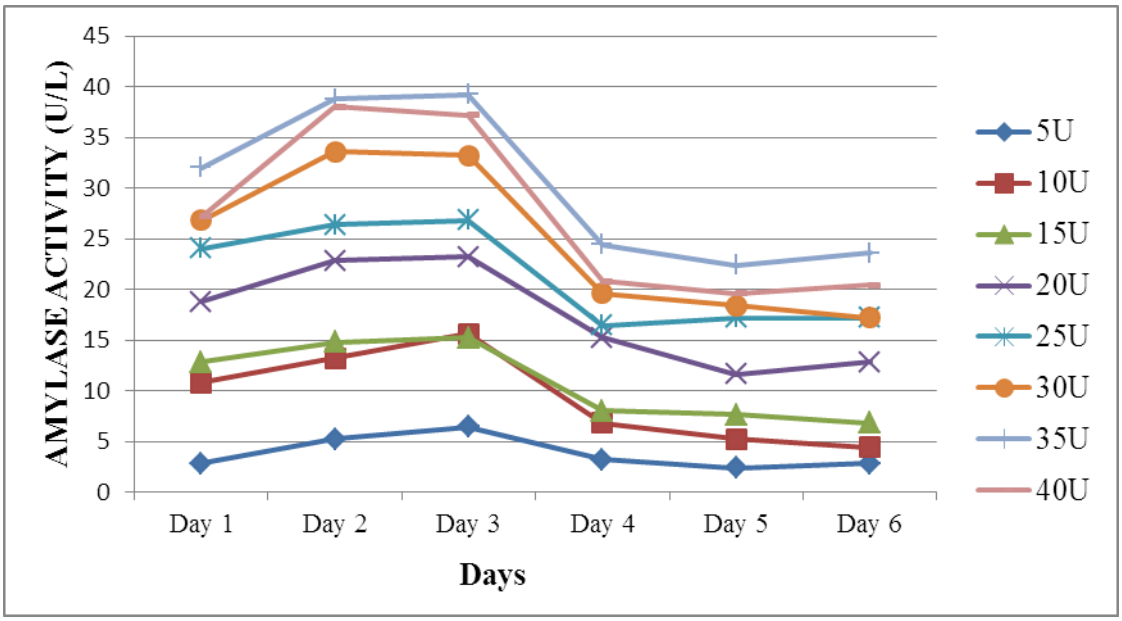

Fig. 3: Biodegradation study of chemically modified egg albumin nanospheres of encapsulated *Cicer arietinum* amylase with different concentration of alkaline protease (5U, 10U, 15U, 20U, 25U, 30U, 35U, 40U).

Applications of Bio-Active Chemically Modified Egg Albumin Nanospheres of Encapsulated *Cicer Arietinum* Amylase in Washing of Stained Cloths

The application of prepared egg albumin nanospheres of encapsulated enzyme with studied alkaline protease units was studied with four different samples of detergent solutions of Ariel, Surf excel, Wheel and Tide to remove ball point pen, white board marker and permanent marker stains form clothes to enhance the efficiency of detergents for washing dry and hard stains from clothes. Because these kinds of tough dried strains used to be not to vanish completely if we washed with these chosen detergent samples only in one wash only. Among the four chosen samples of detergent solutions, Ariel detergent with bio-active mixture of chemically modified enzyme loaded egg albumin nanospheres was found to be best as compared to others chosen detergents samples followed by Surf excel and Wheel and Tide (Fig 4, 5, 6 & 7) whose washing results were fairly comparable to previous results.[18, 25, 26, 27, 29] to reduce labour, cost and time too.

Fig. 4: Washing results of stained cloths with Ariel only and Ariel with chemically modified egg albumin nanospheres of encapsulated *Cicer arietinum* amylase.

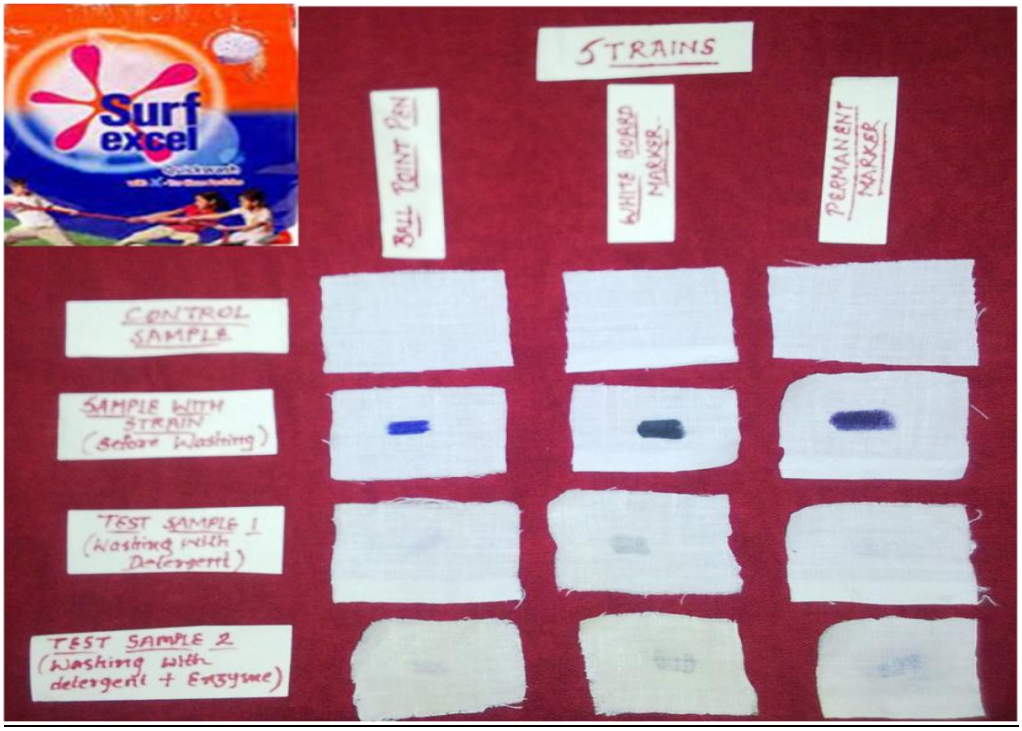

Fig. 5: Washing results of stained cloths with Surf excel only and Surf excel with chemically modified egg albumin nanospheres of encapsulated *Cicer arietinum* amylase.

Fig. 6: Washing results of stained cloths with Wheel only and Wheel with chemically modified egg albumin nanospheres of encapsulated *Cicer arietinum* amylase.

Fig. 7: Washing results of stained cloths with Tide only and Tide with chemically modified bovine egg nanospheres of encapsulated *Cicer arietinum* amylase.

CONCLUSION

Cicer arietinum amylase was encapsulated into chemically modified egg albumin nanospheres with 80% of encapsulation. Size of prepared enzyme loaded egg albumin nanospheres was found to be 101nm to 157nm. The biodegradation study of prepared nanospheres was showed that 35U of alkaline protease was effective for controlled and sustained release of encapsulated enzyme form chemically modified egg albumin nanospheres as well as its bio-active preparation was found to be effective enhancer when used for the removal of stains from cloths having chosen tough dried strains of ball point pen, white board marker and permanent marker when used with chosen detergents (Ariel, Surf Excel, Wheel and Tide). Ariel was found to be the most efficient detergent when used with bio-active enzyme loaded egg albumin nanospheres as detergent echo-friendly additive. Storage stability and thermal stability of encapsulated enzyme was increased up to 6 months and up to 70ºC respectively as compared to free enzyme (24 hours only and 50ºC respectively) with excellent reproducibility. Thus, this enzyme loaded chemically modified egg albumin nanospheres may have fairly good industrial application in beverages industries as a eco-friendly saccharification agent for preparation of fructose syrups and maltose syrups, in paper industries for the treatment of cellulose/ starch, in leather industries for desizing of leather/ various types of natural and synthetic fabrics too as well as may have excellent application in cosmetic, alcoholic/ non-alcoholic/ fizzy drinks/ canned juices beverages, food, pharmaceutical industries, in detergents industries too.

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