Inulin hydrolysis by inulinase immobilized covalently on magnetic nanoparticles prepared with wheat gluten hydrolysates

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A R T I C L E   I N F O

Article history:
Received 13 August 2017
Received in revised form 6 February 2018
Accepted 7 February 2018
Available online 9 February 2018

Keywords:
Inulin hydrolysis
Inulinase immobilization
Magnetic nanoparticles
Wheat gluten hydrolysates
High fructose syrup

A B S T R A C T

Inulinase can produce a high amount of fructose syrup from inulin in a one-step enzymatic process. Inulinase from Aspergillus niger was immobilized covalently on Fe3O4 magnetic nanoparticles functionalized with wheat gluten hydrolysates (WGHs). Wheat gluten was enzymatically hydrolyzed by two endopeptidases Alcalase and Neutrase and related nanoparticles were prepared by desolvation method. Magnetite nanoparticles were coated with WGHs nanoparticles and then inulinase was immobilized on it using glutaraldehyde as crosslinking agent. Parallel studies employing differential scanning calorimetry and field emission scanning electron microscopy were carried out to observe functional and structural variations in free inulinase during immobilization. Optimum temperature of immobilized inulinase was increased, while, pH and Km values were decreased compared to free enzyme. Overall, a 12.3 folds rise was detected in enzyme half-life value after Immobilization at 75 °C and enzyme preserved 70% of its initial activity after 12 cycles of hydrolysis with 75% of enzyme loading.

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1. Introduction

Microbial inulinas belong to an important class of industrial enzymes that have gained increasing attention in the recent years. A series of microorganisms such as fungi, yeasts, and bacteria can produce inulinas [1]. Inulin represents a source for the production of high fructose syrup through enzymatic hydrolysis by either single exo-inulinase (EC 3.2.1.80; β-D-fructofuranosidase) or cooperative action with endo-inulinase (EC 3.2.1.7; β-fructan fructanohydrolase) [2]. This enzyme is stored in the underground parts of chicory, dalia, and Jerusalem artichoke [3]. Inulin as a polysaccharide composed of fructose units appears to be an attractive source of fructose syrup. The use of inulinase has made it possible to obtain pure fructose syrup by one-step enzymatic process instead of using acid hydrolysis of the inulin and prevention of difructose anhydrides formation at low pH of process as well as the costly, uneconomical, and multi-stage enzymatic process for starch hydrolysis performed by α-amylase, glucoamylase, pullulanase, and glucose isomerase [4,5]. Inulin has low solubility and high capability to microbial contamination in water at room temperature. Therefore, industrial hydrolysis of inulin needs to be carried out at higher temperatures since it permits the use of higher inulin substrate concentration due to the increased solubility [6]. Thus, inulin would be hydrolyzed more efficiently by thermostable inulinas as inulinoletic enzymes for food and chemical industry usages. There are many reasons for the immobilization of the produced enzymes such as easy separation of the enzyme from the product, reuse of the enzyme from the reaction medium, enhancement of enzyme stability against pH, temperature, solvents, contaminants, and impurities, ideality for multi-enzyme reaction systems, and ease of controlling enzymatic process [7]. Immobilization of enzymes commonly is accomplished by four methods including physical adsorption of enzyme molecules on a support material, entrapment or encapsulation of the enzyme in polymers, covalent binding to a support (that provides more strong, stable and irreversible linkages compared to other methods), and carrier- free immobilization by cross-linked enzyme aggregates (CLEAs or CLECs) procedure [8,9].

Recently, considerable attention has been paid to immobilization of enzymes on nanomaterials such as nanopolymers, nanofibers, and nanoparticles. In fact, reducing the size of the enzyme...
bonded to the carrier increases mass transfer and improve the efficiency of the enzyme. Smaller particles provide a larger surface to enzyme loading per unit mass of the particles [10].

Using magnetic nanoparticles as a carrier provides the possibility of easy and low-cost collecting of enzyme from the reaction medium by the magnetic field. Moreover, surface modification of magnetic nanoparticles with functional groups is a useful strategy for suitable attachment of the enzyme to the carrier, increasing the stability of nanoparticles against oxidation and acidic environments and reducing the accumulation of nanoparticles to each other [11]. Above that, functional groups such as protein amino acid residues (for instance lysine ε-amino groups) provide magnetic nanoparticles with a more reactive surface for enzyme immobilization.

In the present study, magnetic wheat gluten hydrolysates nanoparticles were fabricated using the co-precipitation and desolvation methods and immobilized inulinase on it through covalent binding via surface lysine ε-amino groups by glutaraldehyde as a crosslinking agent. Then, the properties of the immobilized enzyme compared with free form was studied [12]. The hypothesis is that, wheat gluten hydrolysates (WGHs) nanoparticles can produce an effective coating on the surface of the magnetic iron oxide nanoparticles which, on the one hand, prevents aggregation of magnetic nanoparticles and, on the other hand, has been created an ideal environment for enzyme binding to the carrier and immobilization of the inulinase to provide a new method for inulin hydrolysis.

2. Materials and methods

2.1. Materials

Inulinase from Aspergillus niger (EC 3.2.1.7 and EC 3.2.1.80) was obtained from Sigma-Aldrich. Glutaraldehyde (25% v/v in water) while sodium-potassium tartrate and 3,5-dinitrosalicylic acid (DNS) were purchased from Merck. Inulin (from Chicory roots) was received from Fluka Company (Fluka, Switzerland). Wheat gluten hydrolysates were produced via enzymatic hydrolysis of wheat gluten by Endopeptidases (Alcalase 2.4 L and Neutrase 0.8 L, provided from Novozymes Company) by Food Science and Technology Lab of Department of Chemical Technologies, Iranian Research Organization for Science and Technology (IROST, Tehran, Iran). All other reagents and solvents used were of analytical grade and obtained from Merck and Sigma-Aldrich.

2.2. Methods

2.2.1. Magnetite nanoparticles (MNPs) preparation

Magnetic nanoparticles were prepared by co-precipitation of Fe\(^{2+}\) and Fe\(^{3+}\) salts in 2:1 molar ratio under the presence of nitrogen gas. Ferric and ferrous chloride (FeCl\(_3\)-6H\(_2\)O and FeCl\(_2\)-4H\(_2\)O) in 2:1 molar ratio were dissolved in deionized water at a pH of 9.5 adjusted by adding 1N sodium hydroxide (NaOH) solution. After stirring (150 rpm) for 30 min, the black precipitation of iron oxide was formed in the solution at room temperature under the presence of N\(_2\) gas. The particles were separated by high-speed centrifuge at 13,000 × g for 20 min, washed with deionized water for three times at pH, 8, and dried in hot air oven at 70 °C for 10 h, respectively. FE-SEM analysis was performed for evaluation of the prepared MNPs [13].

2.2.2. Gluten hydrolysates nanoparticles preparation

The wheat gluten hydrolysates (WGHs) nanoparticles were prepared by the desolvation method. Wheat gluten hydrolysates were previously produced by the Department of Food Science and Technology of IROST through the enzymatic hydrolysis of wheat gluten by endopeptidases (Alcalase and Neutrase) in phosphate buffer pH 6.5 at 60°C for 7 h. The molecular weight of gluten hydrolysates specified by the SDS-PAGE method and mostly was between 35 and 45 KDa. For this reason, WGHs were solubilized in 100 ml of deionized water (0.1-1% w/v) and its pH was adjusted to 10 with 1 M NaOH solution under stirring at 500 rpm. Then, ethanol was added dropwise to WGHs aqueous solution with ethanol/water ratio 7:3 and 0.5% (w/v) of Tween-80 was added to the mixture as a surfactant to stabilize the preparation. Afterward, most of the ethanol was eliminated by evaporation with a rotavapor and its final volume was adjusted to 100 ml. Desolvolation method was accomplished by sonication technique (400 Hz, 5 min, 25 ± 5°C) and then large aggregates were separated by centrifuge at 11337 × g for 15 min. Then, the supernatant containing WGHs particles was dispersed by three sessions of 5 min ultrasonic waves. Next, the resulting WGHs nanoparticles were crosslinked with 197 μl of glutaraldehyde (25% v/v in water) and the stirring was kept for 2 h at a room temperature. The particle size, size distribution, and zeta potential of particles were determined by DLS method. Also, the morphology of nanoparticles was specified by FE-SEM technique [14].

2.2.3. Magnetic nanoparticles functionalization by gluten hydrolysates nanoparticles

Magnetic nanoparticles were coated with gluten hydrolysates nanoparticles by adding 0.06 g of prepared Fe\(_3\)O\(_4\) nanoparticles, to 0.05 g of gluten hydrolysates NPs (1:2:1 ratio) with 197 μl of glutaraldehyde (25% v/v) into 25 ml sodium phosphate buffer (pH 7.4). The solution was subjected to ultrasonic waves for 5 min and then stirred at room temperature for 24 h [15].

2.2.4. Immobilization of inulinase on Fe\(_3\)O\(_4\)-Gluten hydrolysates nanoparticles

In this step, 4 ml inulinase enzyme (4.47 mg protein/ml) was added to magnetic gluten hydrolysates nanoparticles solution containing glutaraldehyde and was mixed under a gentle stirring speed at 4°C for 24 h. The covalently immobilized enzyme was centrifuged and washed three times with phosphate buffer solution [3].

2.2.5. Protein content and inulinase activity assay

Protein content was determined according to Bradford's method using bovine serum albumin (BSA) as the protein standard [16]. The activities of free and immobilized inulinas were estimated according to the dinitrosalicylic acid (DNS) method. Soluble inulin (1% w/v) in sodium acetate buffer (pH 5.5) was used as a substrate. The assay mixture contained 100 μl inulinase, 1000 μl of soluble inulin (1%/w/v), and 9000 μl sodium acetate buffer (pH 5.5). The cocktail was hydrolyzed for 0–60 min in a water bath shaker at 38–40°C. The reaction was terminated by adding 500 μl of DNS reagent to 500 μl of hydrolyzed mixture followed by incubating it at 97–98°C for 10 min. The activity was determined (μmol/min \(^{-1}\)) by reading the absorbance intensity at 575 nm using Perkin Elmer, Lambda 25 UV/VIS spectrophotometer against the blank [17].

2.2.6. Effect of pH and temperature on enzyme activity

The optimum temperature regarding enzymatic activity of free and immobilized inulinas were determined in the range from 35 to 75°C. The optimum pH for inulinase activity was tested by incubation of enzyme at 40°C in sodium acetate buffer (pH 5–6) and phosphate buffer (pH 7–8) using inulin suspension (1% w/v) as the substrate. Samples were withdrawn at a determined time and the enzyme activity was determined by DNS method as described previously [18].
Fig. 1. SEM images and Particle size distribution graphs of nanoparticles. Magnetic nanoparticles (a and b), gluten hydrolysates nanoparticles (c and d) and immobilized inulinase on MGHNPs (e and f).
2.2.7. Thermal stability
Thermal stability of the free and immobilized inulinase was evaluated by incubation of the enzyme in acetate buffer (pH, 5.5) without substrate at 45, 55, 65, and 75 °C for 1, 5, 10, 15, 30 and 60 min. Then, the remaining activity was determined by addition of substrate solution (soluble inulin 1% w/v) at 40 °C [18]. The rates of inactivation were calculated by a least-squared fit of plots of the log of the remaining activity against time.

Based on the data obtained by thermal stability study, the inactivation rate constant (k_{in}) (min^{-1}) was determined either as the slope of the linear regression of \( \ln \left( \frac{A_t}{A_0} \right) \) versus time at the constant temperature model (Eq. (1))

\[
\ln \left( \frac{A_t}{A_0} \right) = \ln \left( \frac{A_t}{A_0} \right) - k_{in} \tfrac{t}{2}
\]

That, \( A_0 \) is inulinase activity before incubation, \( A_t \) is inulinase activity after incubation at the temperature of interest and \( t \) is the incubation time.

The half-life of inulinase (\( t_{1/2} \), min) or the time required for losing 50% of initial activity was estimated using Eq. (2) [19,20].

\[
t_{1/2} = \ln 2 / k_{in}
\]

2.2.8. Determination of kinetic parameters
The inulin hydrolysis by inulinase follows Michaelis–Menten kinetics. Thus, the \( K_m \) and \( V_{max} \) values of free and immobilized enzymes were specified by measuring their activities in the presence of inulin as enzyme substrate in concentrations between 0–5 mg/ml in sodium acetate buffer (pH, 6) at 40 °C. For this purpose, \( K_m \) and \( V_{max} \) values of the enzymes were determined using Lineweaver-Burk plots drawn based on the reversed initial velocity against inversed of substrate concentrations [18].

2.2.9. Scanning electron microscopy
A low vacuum Tescan Mira II Field Emission Scanning Electron Microscope (FE-SEM, Czech Republic) was used for morphological characterization of prepared Fe₃O₄ nanoparticles, gluten hydrolysates nanopeptides, and immobilized inulinase on magnetic nanoparticles after coating the samples with a thin layer of gold by magnetron sputtering.

2.2.10. Reusability
The reusability of immobilized inulinase on functionalized magnetic NPs was measured by the hydrolysis of 1000 µl of 1% inulin solution in pH, 5.7 sodium acetate buffer at 40 °C. The DNS assay and samples extraction and testing were the same to those described in “2.2.5 Inulinase activity assay” section. For each cycle, the hydrolysis of inulin by immobilized inulinase took 60 min. After each cycle, the immobilized inulinase was collected by centrifuging at 11337 × g and washed with a phosphate buffer pH of 7.0 for three times and dispersed in a fresh sodium acetate buffer pH of 5.7 for the next cycle. The initial concentration of inulinase solution for immobilization on functionalized magnetic nanoparticles was 7.5 mg of protein per gram of nanoparticles. The first cycle was recognized as the control group, and its activity was defined as remaining activity (100%). In order to study the activity’s decay of immobilized inulinase, 12 cycles hydrolysis of inulin were performed on each sample for 60 min [18].

2.2.11. Enzyme loading
The amount of protein loaded on the support was calculated using the following equation:

\[
Q = \frac{(C_1 - C_2)V}{W}
\]

Where Q is the value of enzyme loading (mg per mg support), \( C_1 \) is the initial loading concentration of enzyme (mg/ml), \( C_2 \) is the enzyme concentration in the supernatant after immobilization (mg/ml), V is the volume of the enzyme solution (ml), and W is the weight of the support. The enzyme content of the supernatant was calculated using the Bradford reagent. To assess the maximum enzyme loading amount, the residual enzyme in the supernatant in the washing stage was considered [21].

3. Results and discussion

3.1. SEM result

The morphology of magnetic nanoparticles, gluten hydrolysates nanoparticles, and immobilized inulinase on functionalized magnetic nanoparticles are compared in Fig. 1. All the prepared magnetic nanoparticles exhibited an approximately spherical shape with 30–40 nm size (Fig. 1a and b). In connection with gluten hydrolysates, after enzymatic hydrolysis and desolvation process of wheat gluten, long filaments of gluten with high mass was converted to nanofibers and spherical nanoparticles with lower mass with a varied size from 140 to 160 nm (Fig. 1c and d). Formation of these water-soluble nanostructures from water-insoluble gluten fibers represents the success of the alteration of protein structure for covering and stabilizing unstable Fe₃O₄ nanoparticles. Besides, SEM image implied that inulinase is covered on the surface of functionalized nanomagnetite via

![Fig. 2.](image-url) Enzyme activity and hydrolysis profile of free and immobilized inulinase. Comparison of enzyme activity of free and immobilized inulinase (a), hydrolysis profile of free and immobilized inulinase (b).
glutaraldehyde (as a crosslinker) with an average size of 160–180 nm (Fig. 1e and f).

3.2. Free and immobilized inulinase activity assay

Due to the covalent attachment of enzyme on support, the relative reduction in enzyme activity after immobilization is expected. As can be seen from Fig. 2a, although immobilized enzyme activity is reduced after immobilization, the persistence of enzyme activity increases and it becomes more active in longer time compared with the free enzyme. In addition, fructose production per unit of time by immobilized inulinase is increased about 35.3% compared to free inulinase (Fig. 2b).

3.3. Effect of pH on enzyme activity

The effect of pH on the relative activity of free and immobilized inulinase at pH range from 5 to 8 is represented in Fig. 3. The optimum pH of the immobilized enzyme for inulin hydrolysis was 5.7, which is slightly lower than that of the free form (pH, 6.0). It seems that, inulinase immobilization via covalent bonding to functionalized nanomagnetic carrier increases the stability of the enzyme in a slightly more acidic environment. This pH shift may be a result of both the charge of the support materials and conformational changes of the enzyme molecules [22].

3.4. The effect of temperature on inulinase activity

The effect of temperature on the activity of free and immobilized inulinase is shown in Fig. 4. The optimum temperature for immobilized enzyme was specified 45 °C, which is 5 °C higher than that of free inulinase. The increase in the structural rigidity and decrease in the flexibility of the immobilized inulinase via covalent bonding can justify the higher optimum temperature of immobilized enzyme compared with free enzyme [23,24].

3.5. Kinetic parameters

The effect of inulin concentrations (0–5 mg/ml) as a substrate on hydrolysis velocity by inulinase was investigated for kinetics assessment. The kinetic constants \( V_{\text{max}} \) and \( K_m \) were estimated by the Lineweaver-Burk plots based on the inverse values of substrate concentrations (1/[S]) and enzymatic reaction velocity (1/V). The resulting kinetic constants are presented in Table 1.

As indicated in Table 1, the \( V_{\text{max}} \) and \( K_m \) values of the free and immobilized inulinase were 0.829 and 2.74, and 0.760 μmol min \(^{-1}\) and 1.88 mg/ml, respectively. As expected, the \( K_m \) and \( V_{\text{max}} \) values of free inulinase decreased after immobilization. The structural changes induced upon immobilization of enzymes on solid supports often yields some modifications of the kinetics compared to the free enzyme. Reduction of \( K_m \) value after immobilization indicates that the substrate tends to be more connected to immobilize enzyme active sites compared to free form and such a slight reduction in \( V_{\text{max}} \) values after immobilization is not unusual. This observation might be explained by the changes in effective localized charges near the active site of an enzyme owing to the proximity between enzyme and support [3].

3.6. Thermal stability and half-life of free and immobilized enzyme

Thermal stability of free and immobilized inulinase was evaluated at 45, 55, 65, and 75 °C respectively. Inactivation rates were calculated by equations 1 and 2 resulted from thermal inactivation of the enzyme species (free and immobilized inulinase). As shown in Table 2, as the temperature rises from 45 to 75 °C, the inactivation rate constants (\( k_{\text{in}} \)) also increases.

Half-life values of the free and immobilized inulinase are shown in Fig. 5 as a function of temperature. Results revealed that, \( t_{1/2} \) values of immobilized inulinase are increased 12.3 folds compared to free inulinase at 75 °C, respectively.

Although \( t_{1/2} \) values decreased with increasing the incubation temperature, the results indicated the higher half-life of

![Fig. 3. The effect of pH on activity of free and immobilized inulinase.](image)

![Fig. 4. The effect of temperature on activity of free and immobilized inulinase.](image)

| \( K_m \) (mg/ml) | \( V_{\text{max}} \) (μmol min \(^{-1}\)) |
|-------------------|----------------------------------|
| Free Enzyme       | 2.74                             | 0.829                          |
| Immobilized Enzyme| 1.88                             | 0.760                          |

| Temperature(°C) | \( K_{\text{in}} \) (10 \(^{-5}\) min) |
|-----------------|----------------------------------|
| 45              | 1.1                              |
| 55              | 2.8                              |
| 65              | 6.8                              |
| 75              | 46.2                             |
immobilized inulinase compared to free enzyme. Therefore, we can claim that thermal stability of inulinase is increased after immobilization on magnetic wheat gluten hydrolysates nanoparticles.

3.7. Reusability

Since it is difficult to separate the free enzyme from the reaction medium and using them frequently, reusability is one of the considerable features of an immobilized enzyme that makes them distinct from free form. Our results show that the immobilized inulinase maintained about 70% of its initial activities after 12 cycles of hydrolysis (Fig. 6).

3.8. Enzyme loading

The results of this study indicated that, the enzyme loading under described immobilization conditions were nearly 75%.

4. Conclusions

In this research, bio-functionalization of magnetic nanoparticles was performed by wheat gluten hydrolysates with an average size between 100 and 160 nm and used as a carrier for covalent immobilization of inulinase. The inulinase immobilized within a broader pH range and a higher optimum temperature might present a better thermal and storage stability. The inulinase loading amounts on fabricated functionalized magnetic nanoparticles were calculated to be 75%.

Enzyme half-life values of immobilized inulinase increased 12.3 folds increment compared to free inulinase at 75°C, respectively. Immobilized inulinase could be successfully reused for 12 cycles with remaining 70% of its initial activity. The hypothesis can be well proven by the stability of immobilized inulinase at low pH, thermal stability, and half-life values compared to the free enzyme and maintaining 70% of its initial activities after 12 cycles of hydrolysis.

Funding

This work was supported by Iranian Research Organization for Science and Technology [grant number 1010291011]; Iran Nanotechnology Initiative Council (INIC) [8532-0097].

Conflict of interest

There is no conflict of interest between authors and their organization, Iranian Research Organization for Science and Technology.

Acknowledgments

The financial support provided by Iranian Research Organization for Science and Technology (IROST) and Iran Nanotechnology Initiative Council (INIC) is greatly acknowledged.

References

[1] E. Ricca, V. Galabró, S. Curcio, G. Iorio, The state of the art in the production of fructose from inulin enzymatic hydrolysis, Crit. Rev. Biotechnol. 27 (3) (2007) 129–145.
[2] W.Y. Kim, S.M. Byun, T.B. Uhm, Hydrolysis of inulin from Jerusalem artichoke by inulinase immobilized on aminoethylcellulose, Enzyme Microb. Technol. 4 (4) (1982) 239–244.
[3] P.K. Gill, R.K. Manhas, P. Singh, Hydrolysis of inulin by immobilized thermostable extracellular exoinulinase from Aspergillus fumigatus, J. Food Eng. 76 (3) (2006) 369–375.
[4] C. Barthomeuf, F. Regerat, H. Pourrat, Production of inulinate by a new mold of Penicillium rugulosum, J. Ferm. Bioeng. 72 (6) (1991) 491–494.
[5] E.J. Vandamme, D.G. Derycke, Microbial inulinases: fermentation process, properties, and applications, Adv. Appl. Microbiol. 29 (1983) 139–176.
[6] H. Torabizadeh, M. Habibi-Rezaei, M. Safari, A.A. Moosavi-Movahedi, A. Sharifizadeh, H. Arzizian, M. Amanlou, Endo-inulinase stabilization by pyridoxal phosphate modification: a kinetics, thermodynamics, and simulation approach, Appl. Biochem. Biotechnol. 165 (7–8) (2011) 1661–1673.
[7] V. Varalakshmi, D. Sivarama Prasad, Enzyme immobilization: the fascinating challenge in biotechnology, J. Environ. Sci. Comput. Sci. Eng. Technol. 2 (3) (2013) 840–853.
[8] J. Missau, A.J. Scheid, E.L. Foletto, S.L. Jahn, M.A. Mazutti, R.C. Kuhn, Immobilization of commercial inulinase on alginate-chitosan beads, Sustainable Chem. Processes 2 (1) (2014) 13.
[9] M. Rosales-Hernández, L. Kispert, E. Torres-Ramírez, D. Ramírez-Rosales, R. Zamorano-Ulloa, J. Trujillo-Ferrara, Electron paramagnetic resonance analyses of biotransformation reactions with cytochrome P-450 immobilized on mesoporous molecular sieves, Biotechnol. Lett. 29 (6) (2007) 915–924.
[10] Y. Jiang, C. Guo, H. Xia, I. Mahmood, C. Liu, H. Liu, Magnetic nanoparticles supported ionic liquids for lipase immobilization: Enzyme activity in catalyzing esterification/Magnetic nanoparticles supported ionic liquids for lipase immobilization: enzyme activity in catalyzing esterification, J. Mol. Catal. B: Enzym. 58 (1) (2009) 103–109.

[11] W. Zhang, J. Qiu, H. Feng, L. Zang, E. Sakai, Increase in stability of cellulase immobilized on functionalized magnetic nanospheres, J. Magn. Magn. Mater. 375 (2015) 117–123.

[12] V. Mozhaev, N. Melik-Nubarov, M. Sergeeva, V. Sikirnis, K. Martinek, Biocatalysis 3 (1990) 179–187.

[13] P.L. Hariani, M. Faizal, D. Setiabuddidaya, Synthesis and properties of Fe3O4 nanoparticles by co-precipitation method to removal procion dye, Int. J. Environ. Sci. Dev. 4 (3) (2013) 336.

[14] C. Duclairoir, E. Nakache, H. Marchais, A.-M. Orecchioni, Formation of gliadin nanoparticles: influence of the solubility parameter of the protein solvent, Colloid Polym. Sci. 276 (4) (1998) 321–327.

[15] Z. Li, L. Qiang, S. Zhong, H. Wang, X. Cui, Synthesis and characterization of monodisperse magnetic Fe3O4@ BSA core–shell nanoparticles, Colloids Surf. A 436 (2013) 1145–1151.

[16] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1–2) (1976) 248–254.

[17] G.L. Miller, Use of dinitrosalicylic acid reagent for determination of reducing sugar, Anal. Chem. 31 (3) (1959) 426–428.

[18] H. Torabizadeh, M. Habibi-Rezaei, M. Safari, A.A. Moosavi-Movahedi, H. Razavi, Semi-rational chemical modification of endo-1,4β-glucanase by pyridoxal 5’-phosphate and ascorbic acid, J. Mol. Catal. B: Enzym. 62 (3) (2010) 257–264.

[19] A. Pal, P. Khanum, Covalent immobilization of xylanase on glutaraldehyde activated alginate beads using response surface methodology: characterization of immobilized enzyme, Process Biochem. 46 (6) (2011) 1315–1322.

[20] X. Zhou, H. Wang, Y. Zhang, L. Gao, Y. Feng, Alteration of substrate specificities of thermophilic α/β hydrolases through domain swapping and domain interface optimization, Acta Biochim. Biophys. Sin. 44 (12) (2012) 965–973.

[21] R.V. Almeida, R.V. Branco, B. Peixoto, C. da Silva Lima, S.M.C. Alqueires, O.B. Martins, D.M.G. Freire, Immobilization of a recombinant thermostable esterase (PZ2001) from Pyrococcus furiosus on microporous polypropylene: isotherms, hyperactivation and purification, Biochem. Eng. J. 39 (3) (2008) 531–537.

[22] S.Z. Mazlan, S. Abu Hanifah, Effects of temperature and pH on immobilized laccase activity in conjugated methacrylate-acrylate microspheres, Int. J. Polym. Sci. (2017) 1–8.

[23] F. Jafari, M. Panjehpour, J. Varshosaz, P. Yaghmaei, Stability improvement of immobilized alkaline phosphatase using chitosan nanoparticles, Braz. J. Chem. Eng. 33 (2) (2016) 243–250.

[24] K. Martinek, A. Klibanov, V. Goldmacher, I. Berezin, The principles of enzyme immobilization I. Increase in thermostability of enzymes covalently bound to a complementary surface of a polymer support in a multipoint fashion, Biochim. Biophys. Acta (BBA)-Enzymol. 485 (1) (1977) 1–12.