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Nanomagnetic wheat gluten hydrolysates a new carrier for nanoimmobilization of inulinase

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ABSTRACT

Functionalized magnetic nanoparticles are effective enzyme carriers since they have several advantages such as easily separation under external magnetic fields as well as reusability of enzymes. In this study, it was tried to prepare a composite nanoparticle with Fe_3O_4 nanoparticles and wheat gluten hydrolysate nanoparticles for utilizing in biocatalyst production. Firstly, Fe_3O_4 nanoparticles were synthesized by chemical co-precipitation. Since these nanoparticles are easily aggregated, surface coating procedure was used. These nanoparticles were coated by wheat gluten hydrolysates (WGHs) using glutaraldehyde as crosslinking agent. WGHs were prepared by enzymatic hydrolysis of wheat gluten by endopeptidases with degree of hydrolysis (24.3%) and then, by desolvation method using ethanol as a desolvating agent. FE-SEM images and DLS analysis showed that Fe_3O_4 nanoparticles have spherical morphology with 30–40 nm in diameter. Also, wheat gluten hydrolysates were ranged 100–150 nm and their zeta potential was around −29 mV. It should be noted that the size of nanocomposite particles were about 120–160 nm. Inulinase was covalently and successfully immobilized through gluteraldehyde on Fe_3O_4@WGH nanoparticles. Moreover, The results implied that, the enzyme was loaded up to 75% onto functionalized nanoparticles.

1. Introduction

The growth of nanotechnology has opened several new possibilities in biology, especially in the field of enzyme and protein immobilization [1]. On an industrial scale applying of recyclable and thermostable enzymes is an important parameter in enzymatic processes as it determines the limits for use and reuse of the enzyme, and therefore affects the cost of final product [2,3]. Size reduction of enzyme carrier improves efficiency of immobilized enzyme. In case of enzyme connection to a surface, smaller particles can provide greater levels to enzymes attachment and as a result, it can increase enzyme loading per unit mass of the particles [4].

Magnetic nanoparticles due to their biocompatibility and superparamagnetic properties, as next generation drug carriers have great attraction in pharmaceutical technology [5]. Moreover, nanomagnetites have attracted much attention as support materials for enzyme immobilization due to their unique magnetic properties, non-toxicity, large surface area to volume ratio, good environmental adaptability, easy control and separation by an external magnetic field [6]. Sustainability of the magnetic nanoparticles for a long time without agglomeration or precipitation is a major challenge. Surface modification of iron oxide nanoparticles with functional groups could help to prevent their aggregation in liquid and improve their chemical stability.

Actually, functional groups such as organic species, surfactants and polymers provide magnetic nanoparticles with a surface friendly for biological systems [7,8]. Functionalized nanoparticles are very promising for applications in liquid-phase catalytic reactions such as enzyme immobilization. Such small, magnetically, separable particles which were coated by organic species such as proteins with lysine amino acid residues are applicable as a functional group for enzyme immobilization [9].

Wheat gluten as an economically important co-product of starch industry, is a high value vegetable protein source. Wheat gluten is actually composed of two different proteins: gliadin (a kind of prolamin protein) and glutenin (a kind of glutelin protein) [10]. Since gluten as a protein macromolecule is viscoelastic and insoluble in near-neutral pH, use of endopeptidases for gluten hydrolysis is an efficient protein modification method to obtain gluten hydrolysates with a high soluble peptide mixtures and much smaller molecular size depending on the degree of hydrolysis [11].

In this study, stable Fe_3O_4 nanoparticles were synthesized by co-precipitation method and were coated by wheat gluten hydrolysate

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(WGH) nanoparticles which are protein nanostructures with high contents amine groups. Peptides derived from enzymatic hydrolysis of gluten and nanopeptides with smaller size, higher solubility and reactivity were used for preparation and functionalization of magnetic nanoparticles by desolvation method.

2. Experimental

2.1. Materials

Wheat gluten was donated from Arian glucose company (Tehran, Iran), Endopeptidases (Alcalase 2.4 L and Neutrase 0.8 L) were provided from Novozyme Company (Novonordisk, Denmark). Ferrous chloride tetrahydrate ($FeCl_2{\cdot}4H_2O$), ferric chloride hexahydrate ($FeCl_3{\cdot}6H_2O$), glutaraldehyde (25% v. in water) were purchased from Merck. Inulinase from *Aspergillus Niger* (EC 3.2.1.7 and EC 3.2.1.80) was obtained from Sigma-Aldrich. Inulin (from Chicory roots) was obtained from Fluka Company (Switzerland). All of the other reagents and solvents were of analytical grade and were obtained from Merck and Sigma-Aldrich.

2.2. Preparation of wheat gluten hydrolysates by endopeptidases (Alcalase and Neutrase)

Wheat gluten (5 g) was mixed with 100 mL of phosphate buffer saline solution at pH = 6.5. An aqueous dispersion of wheat gluten was incubated in a water bath at 60 °C. When temperature of gluten dispersion reached to 60 °C, gluten enzymatic hydrolysis was initiated by adding Alcalase and Neutrase (Alcalase to Neutrase ratio was 2:1) and then, the reaction was continued for 24 h [12].

2.3. Degree of gluten hydrolysis (DH)

Degree of gluten hydrolysis (DH) was determined by trichloroacetic acid (TCA) method. In this way, the percentage of solubilized protein in 10% (w/v) of trichloroacetic acid, in relation to the total protein content of sample, was measured by method of Hoyle and Merritt [13]. 500 µL of hydrolyzed protein was mixed with 500 µL of 20% (w/v) TCA solution to obtain soluble and insoluble fractions in 10% (w/v) TCA solution. After 30 min of rest, the mixture was centrifuged at 3000 rpm and the supernatants were analysed for nitrogen by Bradford method [14]. The result was expressed as milligram of protein. Bovine serum albumin was used as a standard. The DH was calculated according to Eq. (1).

$$DH(\%) = \frac{\text{Soluble protein content in 10 g \% TCA (mg)}}{\text{Total protein content (mg)}} \quad (1)$$

2.4. Preparation of gluten hydrolysate nanoparticles

Wheat gluten hydrolysate (WGH) nanoparticles were prepared by desolvation method. In brief, distilled water was added to 0.1–1% (w/v) WGH to make total volume of 100 mL and then, pH was adjusted to 10 with 1 M NaOH solution under stirring at 500 rpm. In the next step, 70 mL of ethanol was added dropwise to 30 mL of aqueous WGH dispersion and 0.5% (w/v) of Tween 80 as surface-active agent was added to mixture. Most of the ethanol was eliminated by evaporation with a rotary evaporator. Then, the nanoparticles were purified by centrifugation for 15 min at 20000 rpm. The pellets were removed and in order to provide smaller particles, the supernatant containing WGH particles was dispersed by three sessions of 5 min ultrasonic waves. Finally, the prepared particles were cross-linked with 197 µL of glutaraldehyde per 1 g of gluten and kept stirring for 2 h at room temperature [15].

2.5. Synthesis of iron oxide nanoparticles

Fe_3O_4 magnetic nanoparticles were prepared by co-precipitation of Fe^{2+} and Fe^{3+} ions under the presence of nitrogen gas. 8.68 g of $FeCl_3{\cdot}6H_2O$ and 3.25 g of $FeCl_2{\cdot}4H_2O$ were dissolved into 200 mL of deoxygenated distilled water. After stirring for 30 min, black chemical precipitation was achieved under vigorous stirring by adding 10 mL of NH_3 (25%) solution and under the presence of N_2 gas. In the next step, the precipitates were separated by a permanent magnet and washed with deoxygenated distilled water until pH = 8. Finally, Fe_3O_4 magnetic nanoparticles were dried in oven at 60–70 °C [16].

2.6. Immobilization of inulinase on Fe_3O_4-gluten hydrolysate nanoparticles

At first, magnetites were coated with gluten hydrolysate nanoparticles by adding 0.06 g of Fe_3O_4 magnetic nanoparticles, 0.05 g of gluten hydrolysate nanoparticles and a few mL of 25% glutaraldehyde into 25 mL sodium phosphate buffer saline solution (pH = 7.4). The reaction system was mixed for 5 min by ultrasonic waves and then, was stirred at room temperature for 24 h [17]. In the next step, inulinase enzyme (4.47 mg protein/mL) was added to Fe_3O_4@WGH nanoparticles (1.10 mg) containing glutaraldehyde and was mixed under gentle stirring at 4 °C for 24 h. Covalently immobilized enzyme was centrifuged and washed 3 times by buffer solution [18]. The whole process of synthesizing the carriers was illustrated in Scheme 1.

2.7. Enzyme loading

The amount of protein loaded on the support was calculated by the use of Eq. (2) where Q is the value of enzyme loading (mg enzyme 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 enzyme solution (mL) and W is the weight of support. The enzyme content of supernatant was calculated by using Bradford reagent [19].

$$Q = \frac{(C_1 - C_2)V}{W} \quad (2)$$

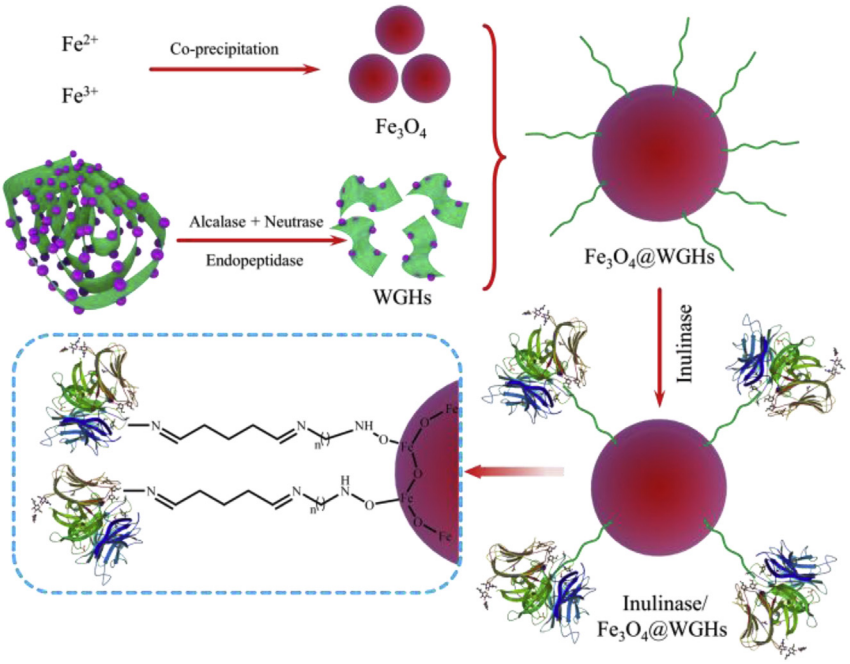
2.8. Characterization

UV–Vis spectroscopy was applied to determine the structural characteristics of gluten hydrolysate nanoparticles and inulinase. (Perkin Elmer Lambda 25 UV/VIS, United States). Fourier transform infrared spectroscopy (FTIR-8300, Shimadzu, Japan) was performed to reveal the surface composition and surface functionality of Fe_3O_4@WGH nanoparticles before and after inulinase immobilization. Fe_3O_4@WGH nanoparticles for FT-IR analysis were prepared in KBr and spectra were recorded in the range 400–4000 cm^{-1}. Dynamic light scattering (DLS) technique was used to determine WGH particle size by measuring the random changes in the intensity of light scattered from WGH solutions with different concentrations (Brookhaven Instrument Corp., USA). Zeta potential as a key indicator of the stability of colloidal dispersions was measured by DLS method for WGH nanopeptides. The morphology of synthesized samples coated with gold were characterized by Tescan Mira II Field Emission Scanning Electron Microscope (FE-SEM, Czech Republic).

3. Result and discussions

3.1. Degree of gluten hydrolysis at different hydrolysis times

According to the procedure mentioned above, wheat gluten was hydrolyzed for 24 h and the degree of hydrolysis (DH) was determined by trichloroacetic acid (TCA) assay. The hydrolytic curve showed that DH values varied and increased from 0 to 24.3% during 24 h of



Scheme 1. Schematic illustration of carrier preparation.

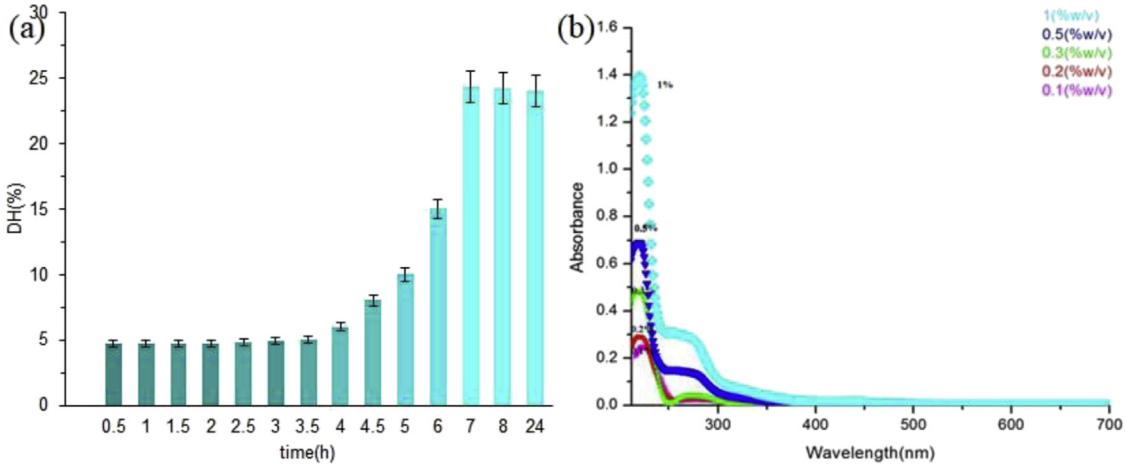


Fig. 1. (A) Degree of hydrolysis for wheat gluten obtained by TCA assay, (b) Absorption curves of WGH in different concentrations (0.1, 0.2, 0.3, 0.5 and 1% w/v).

incubation (Fig. 1a). The highest DH was obtained between 7 and 24 h of hydrolysis. According to the results, gluten was rapidly hydrolyzed during the initial 7 h of hydrolysis but after that, hydrolysis rate did not increase significantly. The sample which was hydrolyzed for 7 h was used for the preparation of WGH nanoparticles.

3.2. UV–Vis spectra of WGH nanoparticles

Commonly, the optical absorbance of protein is measured at 280 nm. At this wavelength, the absorbance of protein is mainly due to the amino acids of tryptophan, tyrosine and cysteine and also, the peptide bond absorbs light in the range of 180–230 nm [20]. UV–Vis spectra of gluten hydrolysates displayed maximum absorption in two wavelength of $\lambda_{max,}$ = 220 and 280 nm (Fig. 1b). Therefore, the results of spectroscopic analysis for WGH nanoparticles confirmed no changes in the protein structure during hydrolysis.

3.3. DLS particles characterization of WGH nanoparticles

DLS analysis of the suspensions of WGH nanoparticles with concentrations of 0.1, 0.2, 0.3 0.5, 1% w/v is illustrated in Fig. 2. WGH suspensions with the lowest concentration (0.1% w/v) was reported to have the smallest average size of nanopeptides (148 nm) as well as the highest zeta potential (−29 mV).

3.4. FE-SEM analysis

By FE-SEM analysis, the information about size and morphology was provided for 0.1% w/v WGH, Fe_3O_4 magnetic nanoparticles and inulinase-magnetic WGH nanoparticles (Figs. 3 and 4). As it can be seen in Fig. 3, Fe_3O_4 nanoparticles were composed of small, uniform and generally spherical particles. Also, particle size distribution demonstrated that Fe_3O_4 magnetic nanoparticles have particle sizes below 50 nm with the average size of about 35 nm. The existence and spatial distribution of Fe and O in magnetic nanoparticles was confirmed by EDX and elemental map analyses.

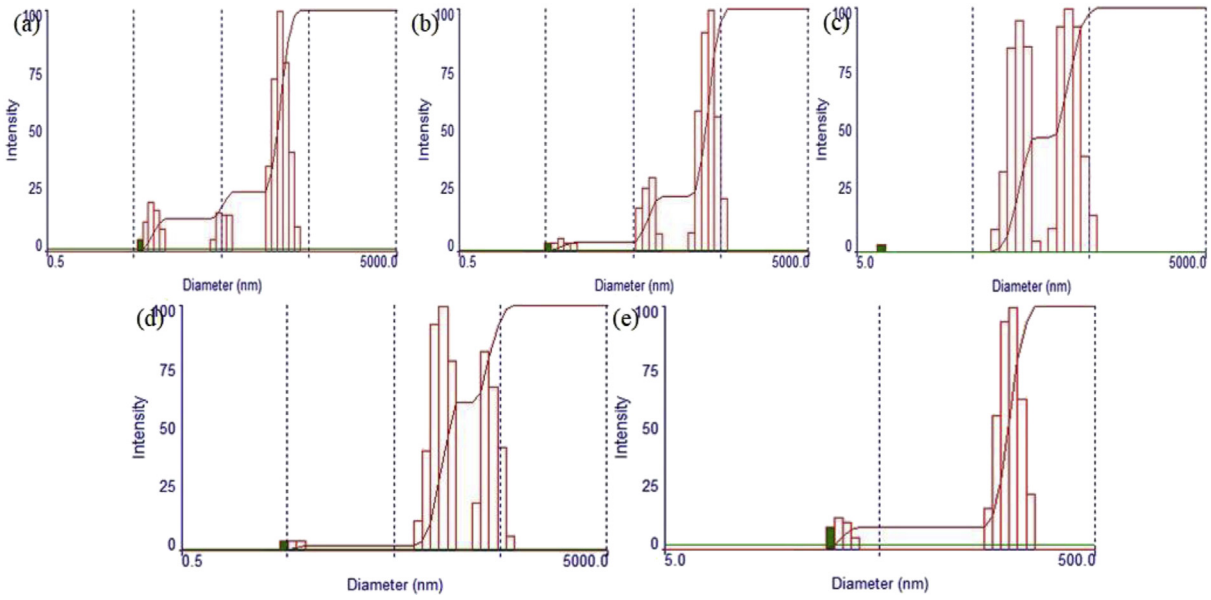


Fig. 2. DLS analysis of WGH nanoparticles with different concentrations, (a) 0.1, (b) 0.2, (c) 0.3, (d) 0.5 and (e) 1% w/v.

Fig. 4a and b showed FE-SEM image and particle size distribution of WGH (0.1% w/v) nanoparticles, respectively. It can be seen that gluten macromolecules with high molecular mass were converted to low-mass segments and were like nanoparticles and or nanofibers. The size of WGH (0.1% w/v) nanoparticles was ranged from 100 to 180 nm. Their common diameter was measured about 140–160 nm (Fig. 4b). Fig. 4c and d illustrates FE-SEM images of inulinase immobilized on Fe_3O_4@GH nanoparticles with two magnifications.

3.5. Fourier transform infrared spectroscopy (FT-IR)

FT-IR spectroscopy was used to identify functional groups before and after inulinase immobilization on the synthesized nanoparticles and confirm correct reactions (Fig. 5). The peak at 578 cm^{-1} was related to Fe-O bond in both un-immobilized and immobilized nanoparticles. However, it should be stated that this peak in modified nanoparticles was slightly overlapped and disappeared. The peaks at 1593 cm^{-1} (N-H bending) and 1292 cm^{-1} (C-N stretching) were indicated for the verification of modification process on Fe_3O_4 nanoparticles with wheat gluten hydrolysate nanoparticles and they can be seen after inulinase immobilization. In the second spectrum, two peaks were appeared. Peak at 1030 cm^{-1} was related to C-O stretching of hydroxyl groups of acetal. It shows that glutaraldehyde reacted well with inulinase. Peak at 1480 cm^{-1} was attributed to C=C bods in the structure of inulinase. It should be noted that existing broad peak at 3150-3600 cm^{-1} in the spectra showed O-H stretching vibrations of hydroxyl functional groups that were coated on the Fe_3O_4 nanoparticles and the peak at 2920 cm^{-1} was for N-H functional groups for immobilized nanoparticles.

3.6. Enzyme loading

Enzyme immobilization appears as a key factor to improve reutilization, thermal stability and reusability [21]. Immobilized enzymes should preserve their structure, function, and their biological activity after immobilization. They should stay tightly bound to the surface of carrier and should not leach during the use of enzyme. It usually eliminates most of the disadvantages for the application of free enzymes

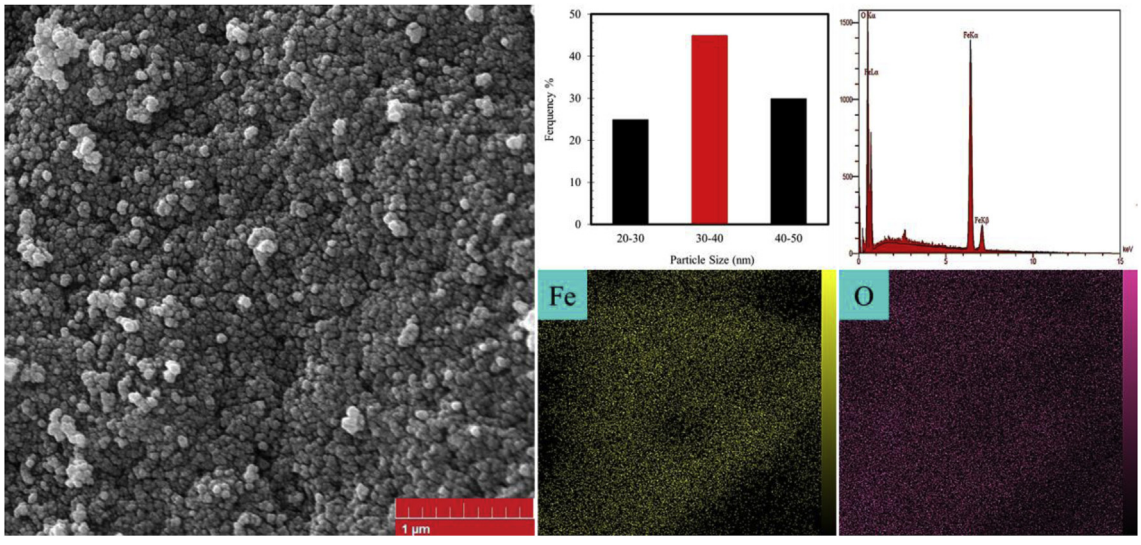


Fig. 3. FE-SEM image, particle size distribution, EDX spectrum and elemental map of Fe_3O_4 nanoparticles.

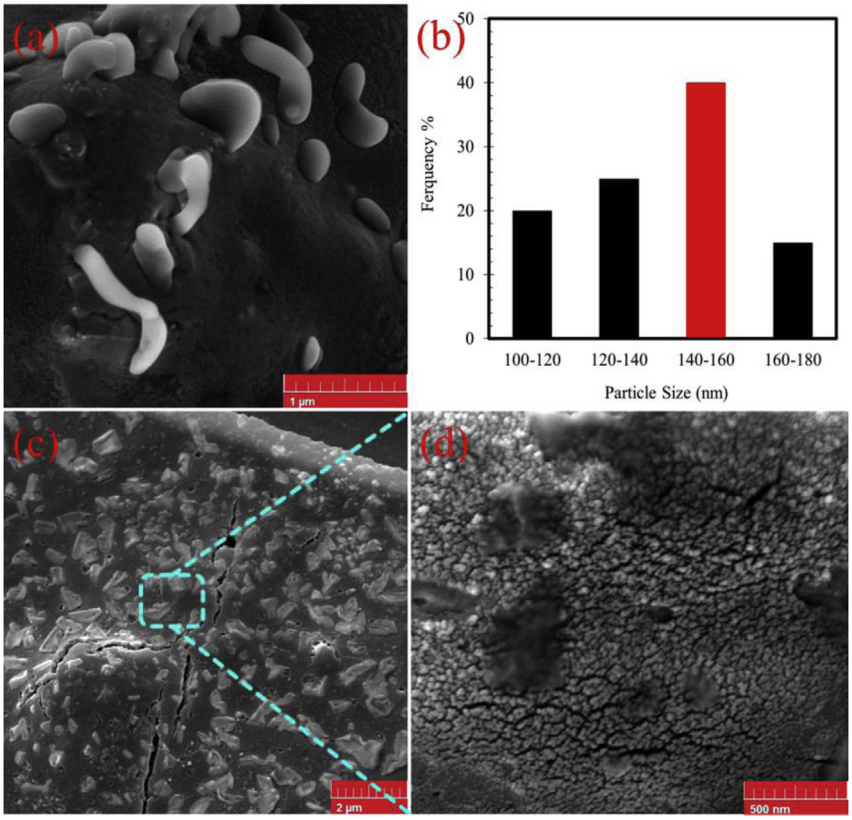


Fig. 4. FE-SEM images (a) and particle size distribution (b) of WGH nanoparticles (0.1% w/v) and (c, d) FE-SEM images of inulinase-magnetic gluten hydrolysate nanoparticles in two different magnifications.

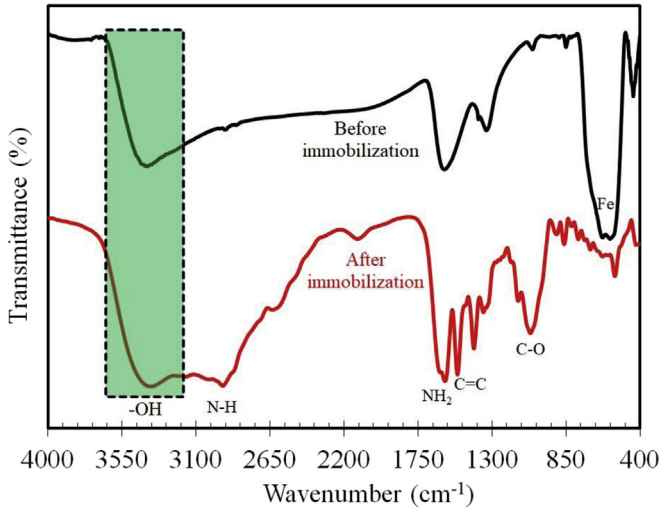


Fig. 5. FT-IR spectra of magnetic nanoparticles before (a) and after (b) inulinase immobilization.

in industrial processes [22]. Nanoparticles provide large surface area per unit mass for high enzyme loading [23]. For enzyme loading results were obtained as follows:

$C_1 = 4.47\ mg\ ml^{-1}$

$C_2 = 1.153\ mg\ ml^{-1}$

$V = 25\ ml$

$W = 1.1\ mg$

Accordingly, the enzyme was loaded under described immobilization conditions (section 2.6.) up to 75% onto functionalized nanoparticles.

4. Conclusions

Wheat gluten was hydrolyzed with Alcalase and Neutrase enzymes during 7 h which it caused to be obtained gluten hydrolysate nanoparticles or nanofibers (148 nm and $\zeta = -29$ mV) by desolvation method. They contained amino acid residues such as lysine or NH_2 groups that had the ability to attach with -OH groups on the surface of magnetic nanoparticles and also, ε-amino group of lysine in the enzyme molecule by using glutaraldehyde in inulinase immobilization. After that, these nanoparticles were coated on Fe_3O_4 magnetic nanoparticles with high surface to volume ratio and high purity (35 nm). Finally, these composite nanoparticles were immobilized by inulinase enzyme. All the analyses were used in this research showed that the reactions were correctly applied. Enzyme loading on wheat gluten hydrolysate-Fe_3O_4 magnetic nanoparticles was around 75%.

Disclosure statement

No potential conflict of interest was reported by the authors.

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