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Papain Immobilization on $NiFe_2O_4$ Magnetic Nanoparticles Functionalized with Gallic Acid and Microwave Assisted Digestion of Bovine Serum Albumin

Nuray Güy*

*Sakarya University, Science and Arts Faculty, Department of Chemistry, Sakarya, Turkey
*nurayg@sakarya.edu.tr

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Abstract

$NiFe_2O_4$ magnetic nanoparticles were solvothermally prepared. $NiFe_2O_4$ magnetic nanoparticles were functionalized with gallic acid and then papain immobilized on $NiFe_2O_4$ functionalized with gallic acid. The obtained samples were characterized and the activities of free and immobilized papain were studied. Immobilized papain showed higher and more effective activity than free papain. This immobilized papain retained about 75.5% of its initial activity after 8 weeks of storage at 4 °C in sodium phosphate buffer (0.1 M, pH 6.5), while the free trypsin protected 30.5% of its initial activity during the same condition. Furthermore, the immobilized papain protected approximately 51% of its initial activity following the times of ten sequential reuse. Finally, the microwave assisted digestion of bovine serum albumin was carried out for 15 s with matrix-assisted laser desorption/ionization mass spectrometry by using free and immobilized papain.

Keywords: $NiFe_2O_4$, gallic acid, papain, immobilization, protein digestion.

1. Introduction

Proteins are enzymatically cleaved into peptide fragments and analyzed using mass spectroscopy (MS). MS/MS sequence analysis and peptide mass mapping are main methods used in protein identification [1]. The in-solution digestion is the most time consuming step in this process. To reduce digestion times immobilization of enzyme onto a solid support has come out as a favorable alternative to the in-solution digestion [1]. The fast and full digestion of all proteins is very important to the productivity and accuracy of protein identification [2]. Papain (3.4.22.2) is an important peptidase which possess a high capacity, hydrolyzing proteins into peptides and amino acids. In addition, papain is used for various applications such as peptide mapping, and production of glycopeptides from purified proteoglycans [3].

Immobilization has been regarded as a beneficial method for protecting enzymes and reusing. The different new kinds of nanoporous and nanoparticles, are currently used to develop immobilized enzyme efficiency. Immobilization of protease enzyme on magnetic support has in recent years been accepted a strong method due to the decreased autolysis products [4].

Magnetic nanoparticles (MNPs) have some advantages as magnetic separation technique such as fast, highly cost-effective, high versatility, environmental friendliness and the reusability [5]. $NiFe_2O_4$ and its nanocomposites have wide application areas, because $NiFe_2O_4$ is a semiconductor, which has magnetic separability and stability [6].

Gallic acid is found both free as part of tannins and react as weak organic acids. It is also widely used in pharmaceutical industry and as an analytical reagent. Gallic acid is highly preferred in biological applications due to its biocompatibility, low cost and high availability [7]. Also, the functionalization of biomacromolecules and its derivatives with phenolic compounds such as gallic acid, tannic acid, and catechin have investigated [8,9].

In this study, for the recycled uses of immobilized papain, the papain coatings were fabricated onto gallic acid modified with $NiFe_2O_4$ MNPs, and successfully employed for bovine serum albumin (BSA) digestion via facile magnetic separation. The digested BSA fragments were identified by MALDI-MS.

2. Materials and Methods

2.1. Materials and apparatus

The solvents and chemicals used in this study were supplied from Merck (Germany) and Sigma-Aldrich (USA) used as received.

The molecular structure of $NiFe_2O_4$ MNPs was verified by X-ray diffraction (XRD, PANalytical, Empyrean). The spectral characterizations were recorded via Fourier transform infrared Shimadzu UATR Two instrument (Japan). Thermogravimetric analysis (TGA) was characterized by Perkin Elmer TGA 4000. Scanning

electron microscope (SEM) images were examined using a Philips XL30 SFEG. Spectrophotometric measurements were performed using Shimadzu UV-2600 UV–Vis spectrophotometer. Mass spectra were obtained in linear modes on a Bruker Daltonics Microflex mass spectrometer (Bremen, Germany) equipped with a nitrogen UV-Laser operating at 337 nm.

2.2. Synthesis of $NiFe_2O_4$ MNPs and modification of gallic acid on $NiFe_2O_4$ MNPs

$NiFe_2O_4$ MNPs were synthesized with using the procedure of Jiao et al. using ethylene glycol (EG) [10].

For the modification of gallic acid on $NiFe_2O_4$ MNPs ($NiFe_2O_4$-GA), the method suggested by Atacan et al. was used in this study [7].

2.3. Papain immobilization and protein assay

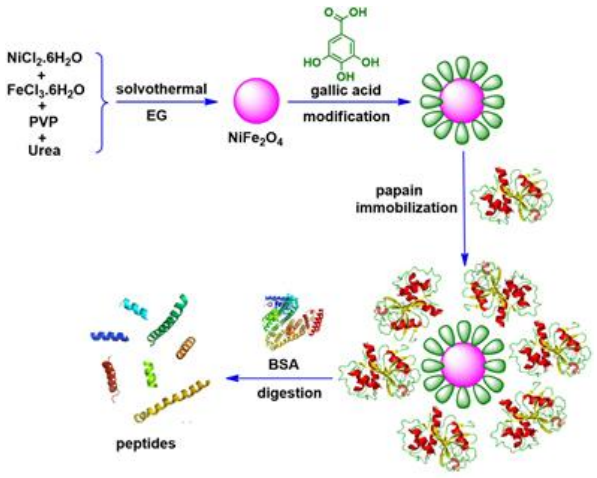
1 mg/mL papain solution (consists of 5 mM L-cysteine in a sodium phosphate buffer solution, PBS, 0.1 M, pH 6.5) was placed on the tube with 0.5 g of $NiFe_2O_4$-GA MNPs. Then the solution was stirred at 250 rpm at 4 °C for 3 h. After 3 h, the immobilized papain ($NiFe_2O_4$-GA-PA) was separated magnetically, and washed with PBS (0.1 M, pH 6.5) for three times [11]. The immobilization process was shown in Scheme 1.

The amount of protein in the papain were determined by the Bradford method [12].

2.4. Papain activity and statistical analysis

The activities of free and immobilized papain were determined via hydrolysis of BAEE at 35 °C by the method described in the studied of Ülkü Metin et al. [13].

All experimental activity studies were measured three times and the results regarded as the mean value (standard deviation (SD) ± 3%).



Scheme 1. The illustration of process of $NiFe_2O_4$ MNPs for BSA digestion.

2.5. Stabilities of papain and reusability

The pH stabilities of free papain or immobilized papain were examined 35 °C for 10 min with different pH (4–10) according to the above mentioned papain activity assay [13]. The determinations of temperature effects of free and immobilized papain were carried out different temperatures (4–80 °C). The free and immobilized papain were stored in its buffer at 4 °C for 8 weeks for storage stability. For the thermal stability, free or immobilized papain was incubated at 60 ºC in PBS (0.1 M, pH 6.5) for 120 min and the residual enzymatic activity was determined under papain activity assay conditions. The reusability of the immobilized papain was detected at 35 °C over ten sequential cycles and the first time detection activity was admitted as 100%.

2.6. Microwave-assisted digestion of BSA

Microwave assisted digestion was studied by the method reported in the studied of Atacan et al., using a microwave oven (CEM-Mars) with a controlled power of 700 W for 15 sec [7]. Finally, two different digestive products were examined by MALDI-MS.

3. Results and Discussion

3.1. Characterizations of $NiFe_2O_4$, $NiFe_2O_4$-GA and $NiFe_2O_4$-GA-PA

Figure 1 shows XRD patterns for $NiFe_2O_4$ MNPs. The characteristic two theta peaks consisting of 18.27°, 30.14°, 35.57°, 37.16°, 43.16°, 53.57°, 57.11°, 62.71° and 74.35° attributed to the crystal planes of (111), (220), (311), (222), (400), (422), (511), (440) and (533), respectively [14]. All diffraction peaks can be regarded as the $NiFe_2O_4$ phase by comparison with the data of ICSD no. 98-018-2237. The result shows that sharp crystalline peaks based on the spinel cubic nickel ferrite [14].

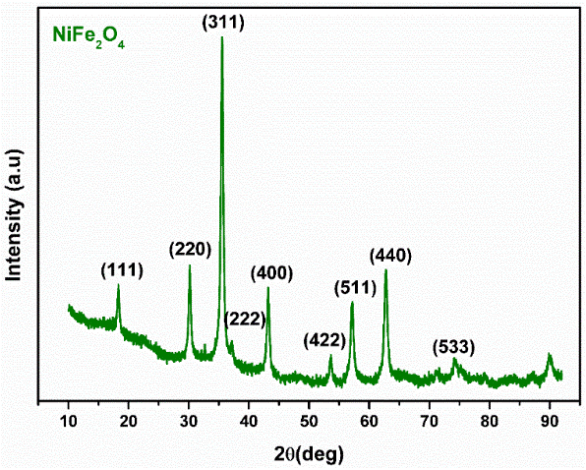


Figure 1. XRD patterns of solvothermally synthesized $NiFe_2O_4$.

The FTIR spectra of $NiFe_2O_4$, $NiFe_2O_4$-GA and $NiFe_2O_4$-GA-PA MNPs are displayed in Figure 2. In the spectrum of synthesized $NiFe_2O_4$ is observed a sharp peak at 554 cm^{-1} to vibration intrinsic of the metal–oxygen (Fe-O)

stretching vibration [15]. $NiFe_2O_4$-GA spectrum demonstrates the absorption bands at 890 cm^{-1}, 1034 cm^{-1} and 1427 cm^{-1}, which comes from structure of gallic acid. The band at 1427 cm^{-1} is ascribed to aromatic C=C bonds. The peak in the range of 2925 cm^{-1} was attributed to the characteristic C–H stretching. The spectrum band at 1648 cm^{-1} corresponded to the C=O vibration of the carbonyl group of amino acid, which shows to amide I band. The spectral shifting of protein amide II band at 1578 cm^{-1} was observed upon enzyme immobilization.

Thermal decomposition behaviors of $NiFe_2O_4$, $NiFe_2O_4$-GA and $NiFe_2O_4$-GA-PA MNPs are shown in Figure 3. In TGA curves, a weight loss of 2% could be ascribed to loss of moisture from 35 to 100 °C [15]. The decrease in weight of $NiFe_2O_4$ was obtained between 100°C and 700 °C of 9.1%, which was caused by the organic residues that remaining in the $NiFe_2O_4$. The weight loss of $NiFe_2O_4$-GA showed about 22.7% until 700 °C which may be functional groups in gallic acid or decomposition of organic remains. $NiFe_2O_4$-GA-PA showed a weight loss of approximately 25% from 100 °C to 700 °C, resulting from thermal decomposition of carboxyl and amine groups in papain.

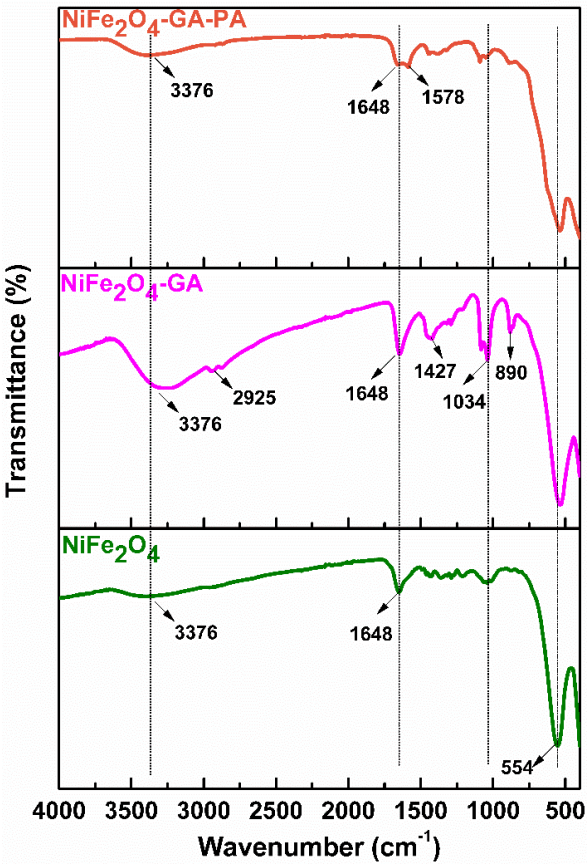


Figure 2. FTIR spectra of $NiFe_2O_4$, $NiFe_2O_4$-GA and $NiFe_2O_4$-GA-PA.

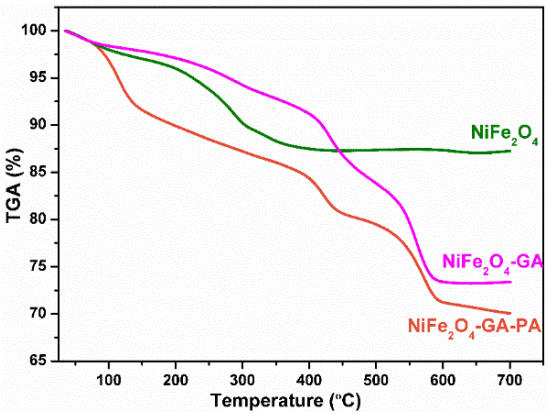


Figure 3. TGA curves of $NiFe_2O_4$, $NiFe_2O_4$-GA and $NiFe_2O_4$-GA-PA.

Figure 4 indicates SEM images of $NiFe_2O_4$, $NiFe_2O_4$-GA and $NiFe_2O_4$-GA-PA MNPs. The $NiFe_2O_4$ (a) is indicating a granular and clustered morphology. The synthesized $NiFe_2O_4$ (a), $NiFe_2O_4$-GA (b) and $NiFe_2O_4$-GA-PA (c) MNPs are completely agglomerated nanoparticles due to electrostatics magnetic attraction [11]. The average grain size obtained from SEM images is in the below 50 nm range.

3.2. The studies of free and immobilized papain activities

The immobilized enzymes are more stable to environmental differences than free enzymes. The pH activity in Fig. 5 (A) pointed out that the immobilization changed the optimum pH (7.5) of papain. The maximum activities of both free and immobilized papain ($NiFe_2O_4$-GA-PA) were observed at pH 6.5 and 7.5, respectively. The $NiFe_2O_4$-GA-PA retained more than 64% of the initial activity even at pH 10.0, whereas the free papain retained about 43%. In addition, the relative activity and pH range of $NiFe_2O_4$-GA-PA were larger than free papain. This pH shift is possibly due to the immobilization. The temperature activities of free and $NiFe_2O_4$-GA-PA were measured from 4 °C to 80 °C according to the mentioned assays of papain activity (Fig. 5 (B)). The maximum activities of both free and $NiFe_2O_4$-GA-PA were obtained at 50 °C. The relative activity of $NiFe_2O_4$-GA-PA was over 52% maintained at 80 °C, whereas free papain was about 26% maintained at the same time. Immobilization of enzymes on solid supports causes the maximum temperature to shift to higher temperatures than free enzymes [16]. The $NiFe_2O_4$-GA-PA has advantage to reuse it thanks to the magnetic nanoparticles since free enzyme could not be reused. The $NiFe_2O_4$-GA-PA retained about 75.5% of its initial activity after 8 weeks. However, the free papain only maintained 30.5% of its initial activity after 8 weeks (Fig. 5 (C)). The thermal stabilities of free papain and $NiFe_2O_4$-GA-PA were measured in different times at 60 °C. Comparing free papain and $NiFe_2O_4$-GA-PA at the same temperature, $NiFe_2O_4$-GA-PA demonstrated higher stability than that of free. $NiFe_2O_4$-GA-PA protected 77% of its initial activity after 120 min at 60 °C, while

free papain protected 34% of its initial activity after the same time and temperature (Fig. 5 (D)). Immobilized enzymes have attracted attention owing to their of advanced reusability. It was monitored that the activity of the $NiFe_2O_4$-GA-PA reduced upon recurring utilizations. The activity of $NiFe_2O_4$-GA-PA was obtained 50.5% after 10 runs (Fig. 5 (E)). The loss in activity was ascribed to the inactivation of enzymes owing to recurring usages. These data show that both the enzymatic activity and stability are well maintained after immobilization. Also, the magnetic immobilized enzymes can be separated easily from medium.

3.3. Digestion of BSA using free and immobilized papain

Scheme 1 describes the schematic of digestion BSA using the prepared $NiFe_2O_4$-GA-PA. Figure 6 (A) and (B) show the MALDI mass spectra of BSA digests obtained using free papain and $NiFe_2O_4$-GA-PA after 15 s reaction. As you can see, the number of identified peptides varied of the Figure 6. For digestion, the BSA was used as a model protein which molecular weight is about 66.5 kDa. BSA is preferred because of its lack of effect in many biochemical reactions and its low cost. The BSA protein that contains 583 amino acids is digested by free papain and $NiFe_2O_4$-GA-PA. They yielded a good spectra by using 3,5-hydroxypbenzoic acid MALDI matrix [17]. BSA was well digested by $NiFe_2O_4$-GA-PA to peptide fragments. Microwave irradiation accelerated and developed the BSA digestion by generating the results of 52% sequence coverage within 15 sec. Compared with free papain, the peptide fragments were detected with a high intensity and 10 peptides can be identified with a sequence coverage of 52% for $NiFe_2O_4$-GA-PA (Fig. 6 B). As illustrated at the higher time intervals in Figure 6, the smaller intensities of peptides with high molecular weight of $NiFe_2O_4$-GA-PA compared to free papain were observed by efficient digestion of BSA. Furthermore, more independent peaks were monitored in the mass spectrum of free papain, which stem from the papain autohydrolysis.

According to the Table 1, Qiao et al., studied BSA digestion for 10 min and 30 min using porous polymer membrane enzyme reactor and they found the identified protein with sequence coverage of 10.3% for 10 min and 89% for 30 min, respectively [18]. Cao et al., found the average sequence coverage of 54.1% for BSA in 15 min using AuNP@Fe_3O_4 enzymatic nanosystem [19]. Ha et al., reported that the sequence coverage obtained from antibody on amine-reactive surfaces was 16% for BSA in 3 hour [20]. This results verified that the microwave-assisted digestion increased the protein digestion with a shorter time interval. The present work shows great potential for magnetic enzyme support applications in fast and influential proteolysis of a small amount of proteins.

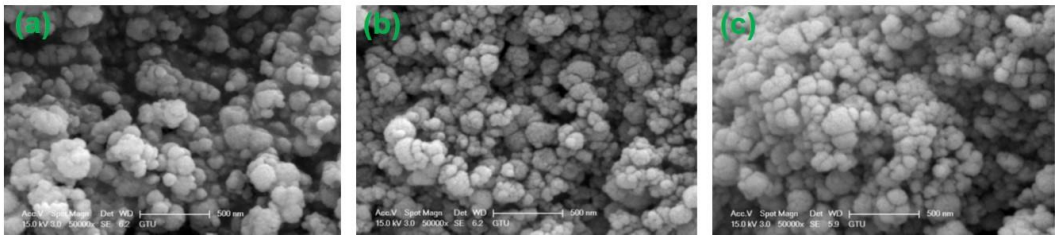
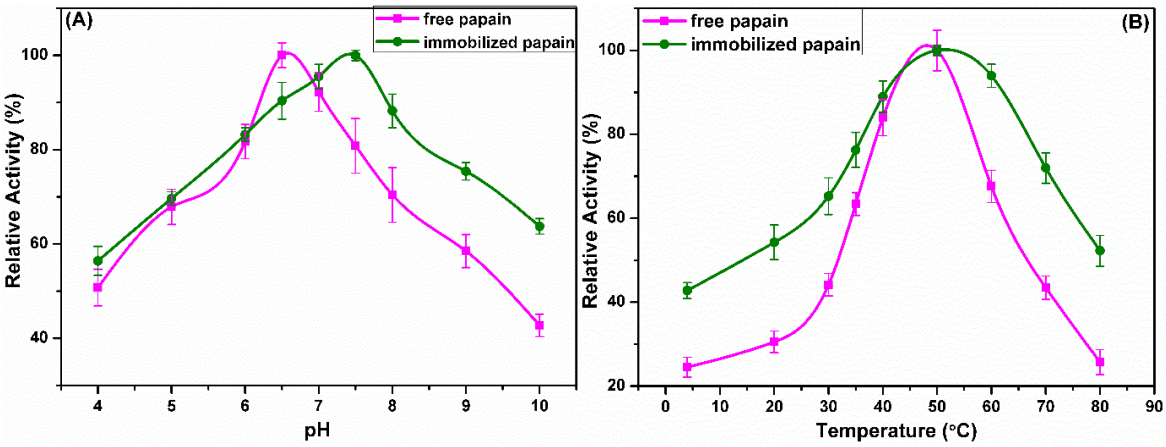


Figure 4. SEM images of (a) $NiFe_2O_4$, (b) $NiFe_2O_4$-GA and (c) $NiFe_2O_4$-GA-PA.



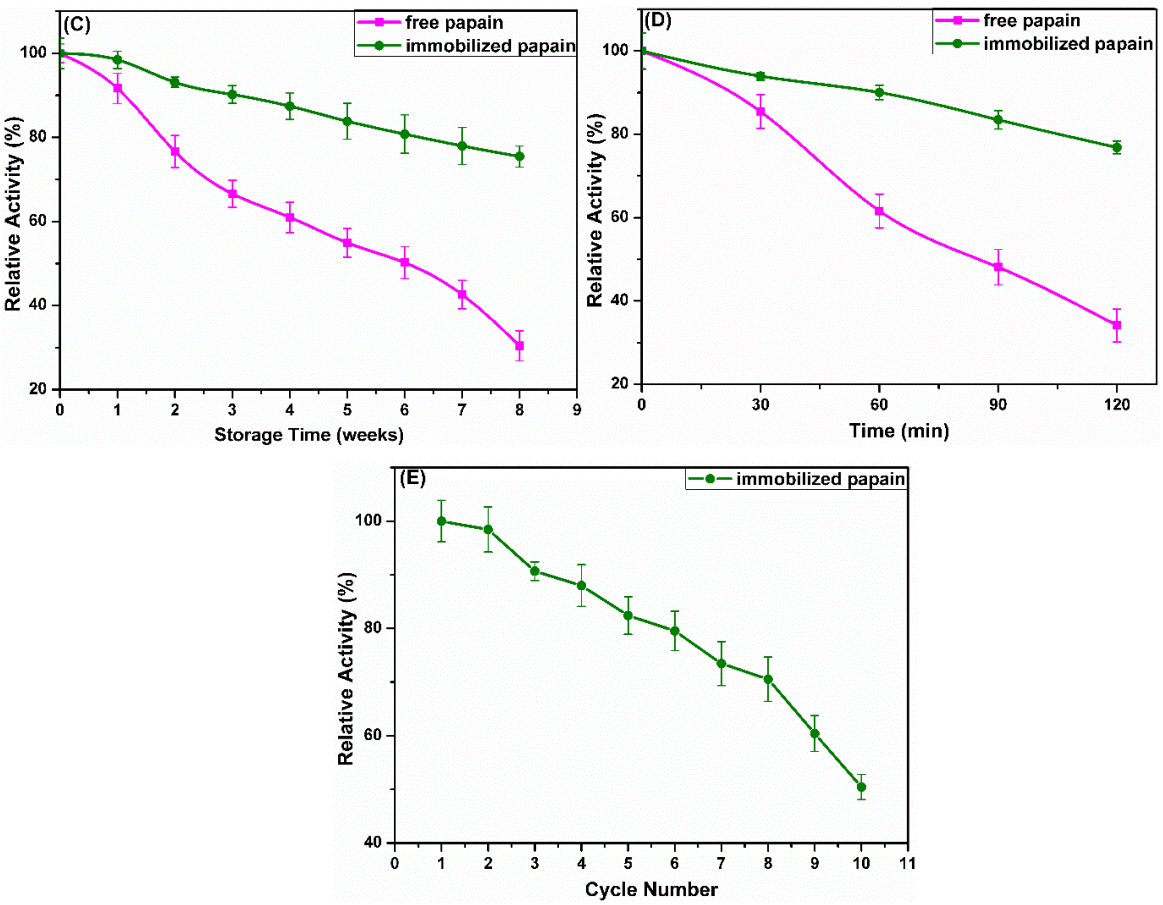


Figure 5. The studies of pH (A), temperature (B), storage time (C) and thermal stability at 65ºC (D) on the activity of free and immobilized papain, reusability of immobilized papain (E) (error bars represents ± standard deviations, n=3).

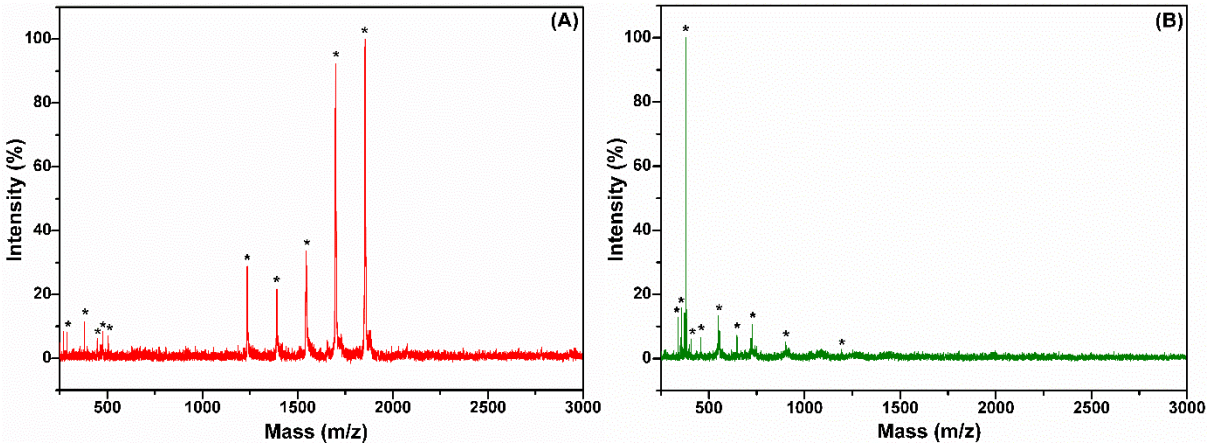


Figure 6. MALDI mass spectra of free papain (A) and immobilized papain (B) from microwave-assisted digestion of BSA for 15 s. * Represents the peptide fragments aquired by the digestion of BSA.

Table 1. Comparison of BSA digestion for this study and other studies.

Support Materials	Digestion method	Digestion Time	Sequence Coverage (%)	References
porous polymer membrane enzyme reactor	-	10 min	10.3	[18]
		30 min	89	
AuNP@Fe_3O_4 enzymatic nanosystem	incubated at 37 °C	15 min	54.1	[19]
Antibody on amine-reactive surfaces	incubated at 37 °C	3 hour	16	[20]
$NiFe_2O_4$-GA-PA	Microwave-assisted	15 sec	52	This study

4. Conclusion

In this study, $NiFe_2O_4$ MNPs synthesized by using solvothermal method and modified with GA, which was non-toxic and biocompatible. Then, papain immobilized on $NiFe_2O_4$-GA MNPs. The determination of activities of free and immobilized papain was carried at the same conditions. BSA was digested using free papain and the prepared $NiFe_2O_4$-GA-PA at the same conditions. The peptide fragments were obtained with immobilized papain higher than free papain. According to the obtained datas, the immobilized papain demonstrated advanced enzyme activity and better to pH and temperature alterations, showing the prepared $NiFe_2O_4$-GA-PA MNPs would be potential application in biocatalysis, biomaterials, potential industrial and medical applications.

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