IMMOBILIZATION OF CHYMOTRYPSIN ON MAGNETIC Fe_3O_4 NANOPARTICLES

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Received May 28, 2013; accepted in revised form September 5, 2013

Abstract: Modern methods of chemical modification of enzymes conferring increased catalytic activity and stability to these molecules have been considered. The advantages of using magnetic nanoparticles for the production of stable immobilized enzyme preparations are presented. Chymotrypsin immobilization on Fe_3O_4 nanoparticles modified with amino groups has been found to result in the incorporation of 88% of the enzyme into the solid phase. The change of the optimal pH and temperature ranges and an increase of stability of the immobilized chymotrypsin relatively to the respective characteristics of the native enzyme have been demonstrated.

Key words: catalytic activity, magnetic nanoparticles, chymotrypsin, glutaraldehyde method, immobilization

UDC 663.15:620.3
DOI 10.12737/2060

INTRODUCTION

The possibilities of application of enzymes, especially in medicine and food processing, have expanded significantly due to the recent advances in enzymology. This is due to the obvious advantages of enzymes over chemical catalysts, namely, selectivity and stereospecificity of action, the ability to attain high substrate conversion rates under relatively mild technological conditions, and safety for the environment and humans [1, 2].

Most enzymes used in food processing are present in food and are ingested with fresh fruit and vegetables, nuts, milk, and fermented and canned foods. The search for new applications of enzymes in various fields of biotechnology is currently going on. The main areas of research include modification of the properties of individual enzymes in order to increase their activity and reduce the cost of the end products, screening of novel enzyme-producing microorganisms, generation of novel recombinant enzymes with desired properties, the use of enzymatic reactions for the production of valuable food ingredients and bioactive substances, and the development of enzyme-based nanotechnology procedures for food processing [2].

The modern methods of enzyme modification confer increased resistance to a variety of chemicals and inhibitors, as well as to pH and temperature effects, to these molecules and allow for alteration of the pH optimum, substrate specificity, and binding properties of the enzymes. Moreover, the catalytic properties of enzymes and the preference of these biocatalysts towards certain metal cofactors can be regulated by modification [2].

Chemical modification of enzymes is among the most widely used methods [3, 4]. The chemical modification procedures must meet a number of requirements. Firstly, the method should employ non-hazardous chemical reagents, especially in case of enzymes subsequently used in food industry. Secondly, harsh conditions of modification leading to enzyme deterioration should be avoided. Thirdly, separation of the modified enzymes from the reaction medium by relatively simple and inexpensive methods should be possible. Finally, the use of the modified enzymes should be cost-effective [4].

The use of non-polar reaction media is an example of chemical modification of enzymes [5]. The resulting reduction of water activity in the reaction system leads to substantial changes of the properties of enzymes, namely, the reaction is shifted towards synthesis, the thermal stability and the storage stability of the enzyme increase, the enzyme acquires an ability to catalyze novel reactions not occurring in an aqueous environment and retains activity in organic solvents at a temperature above 100°C. This method of chemical modification is applicable for such enzymes as lipase, chymotrypsin, trypsin, subtilisin, thermolysin, polyphenol oxidase, glucoamylase, papain, and chymosin.

Research on biological methods of enzyme modification is an actively developing area of enzymology. Protein engineering is an especially promising approach. The methods of protein engineering based on information on the relationship between amino acid sequence, three-dimensional structure, and catalytic activity of enzymes allow for successful modification of enzymes resulting in improvement of their technological characteristics [6, 7]. Substitution of certain amino acid residues in the enzyme molecule is a widely used method.

Substitution of amino acid residues in the enzyme molecules can be used to alter the substrate specificity of these biocatalysts [8]. For example, the ratio of cellobiohydrolase activity towards soluble and insoluble substrates can be altered by replacing the external aromatic amino acid residues which bind to the end of the polysaccharide molecule and direct it into the active site. Resistance of the enzyme to high temperature and extreme pH values is achieved by replacing pairs of amino acid residues located close to each other in the tertiary structure of the enzyme in order to create additional non-covalent hydrophobic bonds, salt bridges, or cova-

lent S-S bonds conferring higher general stability to the globular enzyme molecule.

Chymotrypsin is an enzyme that belongs to the hydrolase class and preferentially cleaves bonds formed by aromatic amino acids (tyrosine, phenylalanine, and tryptophan) in proteins and peptides. Chymotrypsin is among the enzymes most frequently used in various areas of biotechnology, including food industry. Diverse approaches to the regulation of the catalytic activity of this enzyme, including changing the degree of hydration in systems of hydrated reverse micelles formed by surfactants [9], use of nonpolar solvents, adsorption, retention in porous matrices, covalent binding, electrochemical polymerization, etc., have been reported. However, these approaches mostly result in conformational changes manifested as reduced proteolytic activity and increased Michaelis constant of chymotrypsin.

Enzyme immobilization on various organic and inorganic carriers, both natural and synthetic, is among the approaches most widely used in studies described above. This method is universal due to the simplicity of the techniques applied, uniform distribution of the enzyme in the bulk carrier, stability of the immobilized preparations obtained, and good reproducibility of their analytic characteristics. Moreover, attachment of enzymes to solid supports allows for a significant improvement of the mechanical properties of enzyme preparations.

A large variety of carriers for the immobilization of biomolecules is available; however, researchers have been recently showing considerable interest towards nanoparticle carriers [10, 11] due to the changes in a range of fundamental properties of matter upon the transition to the nano-sized state. The large surface of the nanosized objects provides for the predominance of surface phenomena and therefore it is one of the main factors determining the physical properties of nanoscale objects. Since the size of nanoparticles is comparable to those of cells, viruses, proteins, and DNA, these particles can approach biological objects, bind to them, and interact with them.

Nanotechnology is currently recognized as a priority direction of research worldwide since it integrates the cutting-edge achievements of physics, chemistry, and biology. The potential of this discipline is enormous, and its realization affects every aspect of human life. Development of approaches to precise manipulation of the functioning of living systems on the subcellular level is a key issue of modern research in the field of nanobiotechnology.

According to the general concepts outlined above, the aims of the present study included optimization of the conditions for the production of a highly efficient and stable chymotrypsin preparation with Fe_3O_4 nanoparticles as the carrier, as well as the assessment of the perspectives and reasonability of its use in various areas of biotechnology.

RESULTS AND DISCUSSION

The presence of a sufficient amount of active groups on the surface of the carrier is a prerequisite for successful enzyme immobilization. However, the surface of Fe_3O_4 nanoparticles is virtually devoid of reactive groups which could be used for covalent binding of the enzyme, and therefore chemical modification prior to enzyme immobilization is necessary. Various methods for modifying Fe_3O_4 nanoparticles have been reported, including alkylation, acetylation, amination, silanization, coating with polysaccharides or polyaniline, etc. Covalent immobilization of chymotrypsin can be performed using glutaraldehyde which joins the amino groups on the surface of the modified nanoparticles to the amino groups of the enzyme, acting as a spacer.

Crystalline α-chymotrypsin (Sigma, the United States) with an activity of 40–60 U/mg, 70% glutaraldehyde and L-tyrosine (99.9 %) from Sigma-Aldrich-Louis (the United States), sodium caseinate (protein content 92% by mass) kindly provided by the Belka company (Moscow, Russia), and other reagents of the "chemically pure" grade manufactured in Russia were used in the present work.

Fe_3O_4 nanoparticles were prepared using the procedure of Massart [12] by treating a 1:2 mixture of ferrous and ferric chloride solutions (concentration 0.25 M) with a 30% (m/m) NH_4OH solution at room temperature. The resulting precipitate was heated to 80ºC for 30 minutes under constant stirring and washed several times with water and ethanol, and afterwards the Fe_3O_4 nanoparticles were separated from the supernatant using a magnetic separator and dried under vacuum at a temperature above 70ºC [15]. The reaction of Fe_3O_4 formation can be schematically represented as follows:

$$FeCl_2 + 2FeCl_3 + 8NH_3 \cdot H_2O \rightarrow Fe_3O_4 + 8NH_4Cl + 4H_2O$$

Surface modification of the nanoparticles was performed by incubating them with urea and dimethylformamide at a temperature of 300°C. Urea was used for the amination of the surface. Afterwards, the Fe_3O_4 nanoparticles modified with amino groups were washed with distilled water several times for complete removal of impurities and dried at 25°C. The washed nanoparticles were equilibrated with 0.1 M phosphate buffer (pH 7.4) and incubated with 25% glutaraldehyde for two hours at room temperature with vigorous stirring. After the incubation the nanoparticles were washed several times with distilled water.

Chymotrypsin immobilization was carried out by adding the enzyme to a suspension of modified nanoparticles in 0.1 M phosphate buffer (pH 7.4). The enzyme which was non-covalently bound to the particles was removed by three washes with distilled water, a 1% aqueous solution of sodium chloride, and a 1% alcohol solution of sodium chloride. The immobilized chymotrypsin preparation was dried at 4°C and stored as a suspension in 0.1 M phosphate buffer at 4°C.

Estimation of the amount of the immobilized enzyme was based on the difference between the protein concentrations in the reaction mixture before and after incubation of chymotrypsin with Fe_3O_4 nanoparticles. The protein content was determined by the Dumas method based on measuring the thermal conductivity of molecular nitrogen generated after combustion of the test sample at a temperature of about 1000ºC in an atmosphere of oxygen and subsequent complete reduction of nitrogen oxides with copper using the protein nitrogen analyzer RAPID N Cube (Elementar, Germany).

The proteolytic activity of the native and immobilized enzyme was assessed according to the modified method of Anson (GOST (State Standard) 20264.2-88 "Enzyme preparations. Methods for assaying proteolytic activity") based on the determination of the rate of enzymatic hydrolysis of sodium caseinate by the enzyme preparation investigated and colorimetric quantitation of the peptides and amino acids formed using the Folin reagent.

The results of the measurements were processed using the methods of mathematical statistics.

The experiment showed that 88% of chymotrypsin was incorporated in the solid phase after six hours of incubation with Fe_3O_4 nanoparticles modified with amino groups in the presence of glutaraldehyde (Fig. 1). The reaction conditions (pH, temperature, composition of the buffer for immobilization, etc.) were optimized in order to attain maximal proteolytic activity of the immobilized enzyme.

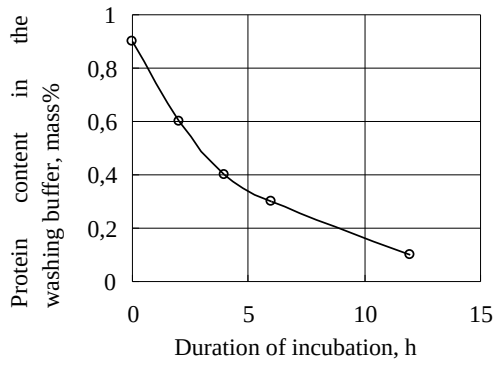

Fig. 1. The dependence of the mass concentration of the protein in the contact solution on the duration of immobilization.

The optimal pH and temperature ranges are important characteristics of the catalytic activity of enzymes. Therefore, investigation of the effect of chymotrypsin immobilization on the optimal functioning conditions of the enzyme was one of the objectives of the present study. Both native and immobilized enzyme were shown to exhibit maximum catalytic activity at pH 8.0 (Fig. 2), but the optimal pH range was much broader for the immobilized enzyme than for native chymotrypsin. The optimal temperature for the immobilized chymotrypsin differed from that of the native chymotrypsin by 5ºC (Fig. 3).

Investigation of the stability of the immobilized enzyme preparation suspended in 0.1 M phosphate buffer (pH 7.4) and stored in a dry, dark place at a temperature of (4 ± 2)ºC (Fig. 4) revealed the decrease of catalytic activity of both native and immobilized chymotrypsin during storage. Native chymotrypsin completely lost activity after 20 days of storage, while chymotrypsin attached to nanoparticles modified with amino groups retained 20% of the catalytic activity after 25 days of storage. Therefore, immobilization of chymotrypsin on Fe_3O_4 nanoparticles results in stabilization of the enzyme.

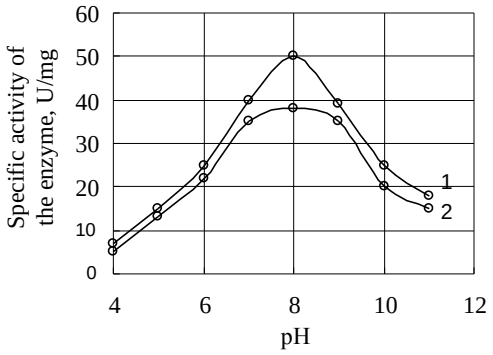

Fig. 2. The pH dependence of the specific activity of chymotrypsin: 1 – native enzyme; 2 – immobilized enzyme.

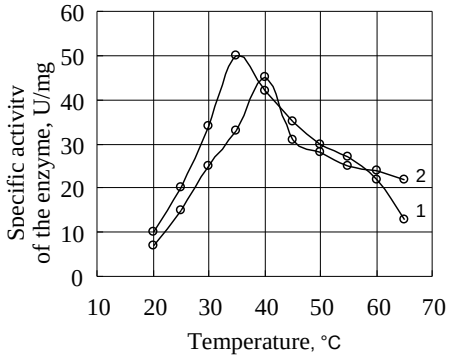

Fig. 3. The temperature dependence of the specific activity of chymotrypsin: 1 – native enzyme; 2 – immobilized enzyme.

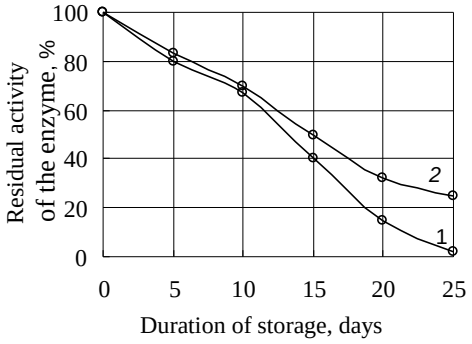

Fig. 4. The dependence of the catalytic activity of chymotrypsin on the duration of storage:1 – native enzyme; 2 – immobilized enzyme.

CONCLUSIONS

Covalent binding of chymotrypsin to surface-modified nanoparticles allows for the production of immobilized preparations possessing high activity and stability; these preparations can be used in different

fields of biotechnology. Besides, the properties of enzyme preparations can be regulated in a controllable manner according to the specific biotechnological tasks by varying the conditions of immobilization.

REFERENCES

1. Volova, T.G., *Biotekhnologiya* (Biotechnology), Novosibirsk: Izdatel'stvo SO RAN, 1999.
2. *Biotekhnologiya* (Biotechnology), Higgins, I., Best, D., and Jones, G., Eds., Moscow: Mir, 1988.
3. Eggins, B., *Khimicheskie i biologicheskie sensory* (Chemical sensors and biosensors), Moscow: Tekhnosfera, 2005, no. 3, pp. 25–31.
4. Gracheva, I.M., *Tekhnologiya fermentnykh preparatov* (Technology of enzyme preparations), Moscow: Agropromizdat, 1985.
5. Antipova, L.V., *Primenenie fermentov v pererabotke vtorichnogo molochnogo syr'ya* (The use of enzymes in the processing of secondary raw materials of dairy origin), Moscow: AgroNIITEIMMP, 1992.
6. Andreeva, I.N., Pantyukhin, A.V., and Sysuev, B.B. *Immobilizatsiya fermentov i drugikh biologicheski aktivnykh soedinenii. Uchebnoe posobie* (Immobilization of enzymes and other biologically active substances. Study guide), Pyatigorsk: Pyatigorskaya Gosudarstvennaya Farmatsevticheskaya Akademiya, 2001.
7. Berezin, I.V. *Biotekhnologiya. Uchebnoe posobie dlya vuzov. T. 7. Immobilizovannye fermenty* (Biotechnology. Student training manual. Vol.7. Immobilized enzymes), Moscow: Vysshaya Shkola, 1987.
8. Berezin, I.V., Klesov, A.A., and Shvyadas, V.K., *Inzhenernaya enzimologiya* (Engineering enzymology), Moscow: 1987.
9. Dixon, M. and Webb, E., *Fermenty. Tom 1,2* (Enzymes. Vols. 1, 2), Moscow: Mir, 1982.
10. Berry, C., and Curtis, A., Functionalization of magnetic nanoparticles for applications in biomedicine, *J. Phys. D. Appl. Phys.*, 2003, vol. 36, pp. R198–R206.
11. Gu, H., Xu, K., and Xu, C., Biofunctional magnetic nanoparticles for protein separation and pathogen detection, *J. of the American Chemical Society Chem. Commun.*, 2006, pp. 941–949.
12. Massart, R., Preparation of aqueous magnetic liquids in alkaline and acidic media, *IEEE Trans. Magn.*, 1981, vol. 17, no. 2, pp. 1247–1248.

The present study was carried out within the framework of the Federal Target Program "Research and research-pedagogic personnel of innovative Russia" for 2009–2013