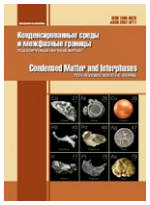

ISSN 1606-867X (Print)
ISSN 2687-0711 (Online)
Condensed Matter and Interphases
Kondensirovannye Sredy i Mezhfaznye Granitsy
https://journals.vsu.ru/kcmf/

Original articles

Research article
https://doi.org/10.17308/kcmf.2023.25/11098

Biocatalysts based on papain associates with chitosan nanoparticles

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Abstract

The research purpose was to develop and study biocatalysts based on papain associates with chitosan nanoparticles. We obtained medium and high molecular weight chitosan nanoparticles, both with and without ascorbic acid .

When the papainna-noparticles complexes with ascorbic acid were formed, the catalytic activity of the enzyme increased by 3 % for medium molecular weight chitosan and by 16 % for high molecular weight chitosan. After 168 hours of incubation in 0.05 M of Tris-HCl buffer (pH 7.5) at 37 °C, the free enzyme retained 15 % of its catalytic activity, whereas its associates with chitosan nanoparticles exhibited ~ 30 %. The papain complex with chitosan nanoparticles and ascorbic acid exhibited 40 % of the enzyme catalytic activity.

We simulated the bonds and interactions within the chitosan-ascorbic acid-papain complex. The proposed biocatalysts have high prospects for effective use in cosmetology, biomedicine, and pharmacy.

Keywords: Nanoparticles, Papain, Chitosan, Association

Acknowledgements: The study was supported by Russian Science Foundation grant (project No. 21-74-20053, complexation of nanoparticles with enzymes).

For citation: Goncharova S. S., Redko Yu. A., Lavlinskaya M. S., Sorokin A. V., Holyavka M. G., Kondtatyev M. S., Artyukhov V. G. Biocatalysts based on papain associates with chitosan nanoparticles. *Condensed Matter and Interphases*. 2023;25(2): 173–181. https://doi.org/10.17308/kcmf.2023.25/11098

Для цитирования: Гончарова С. С., Редько Ю. А., Лавлинская М. С., Сорокин А. В., Холявка М. Г., Кондратьев М. С., Артюхов В. Г. Биокатализаторы на основе ассоциатов папаина с наночастицами хитозана. *Конденсированные среды и межфазные границы*. 2023;25(2): 173–181. https://doi.org/10.17308/kcmf.2023.25/11098

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1. Introduction

Nanoparticles are highly dispersed, usually spherical particles with dimensions not exceeding 100 nm. Nanoparticles have unique properties that make them useful for biomedical applications. The use of polymeric nanoparticles as carriers of genes and drugs is promising, as they are capable of controlled release and targeted delivery of biologically active substances [1].

Nanoparticles have different properties from those of macromolecules. They have a high specific surface area, causing an increase in dispersion. It influences the rate and the ability of the drug to be absorbed by the body systems. Due to the large interface area, nanoparticles are characterised by high drug sorption, which contributes to them more efficient use [2, 3]. Nanoparticles are energy-intensive systems:molecules or atoms on the interface lead to excess surface energy. To minimise this , nanoparticles interact effectively with any compounds and bind rapidly to each other.

The properties of nanoparticles are "collective", they are determined not by a single particle, but by an ensemble of particles distributed in the dispersion medium. Therefore, the characteristics of the microenvironment are a determining factor in the drug delivery properties [4].

One of the promising materials for targeted delivery systems is chitosan. It is a modified natural polyamino-β-glycoside that has biodegradable, antibacterial, and antifungal properties [5–7]. The polymer is highly mucoadhesive and non-immunogenic [8].

Proteases are applied in many industrial processes, for example, in the food and pharmaceutical technologies, as well as in medicine. Papain has a special place among the frequently-used plant proteases [9, 10].

Papain (EC 3.4.22.2) is a proteolytic enzyme isolated from the unripe papaya peel (*Carica papaya*). The enzyme belongs to the cysteine protease family, it is stable under a wide range of conditions, even at high temperatures and pH values of 3–12. Papain has antibacterial, antioxidant, and antitumour properties. Its complexes are used as a pharmaceutical adjuvant [11–14].

The main disadvantage of proteolytic enzyme soluble forms is their rapid inactivation due to proteolysis. One of the ways for increasing the stability of proteases is their association with nanoparticles.

Therefore, the aim of this work was to develop biocatalysts based on papain associates with chitosan nanoparticles and study their catalytic activity.

2. Experimental

Papain was the focus of the study and azocasein (Sigma, USA) was used as a substrate for hydrolysis. Nanoparticles were obtained from medium molecular weight (200 kDa) and high molecular weight (350 kDa) chitosan (Bioprogress, Russia).

Chitosan nanoparticles were obtained as follows: 300 mg of chitosan was dissolved in 100 ml of a 0.3 % acetic acid solution under mechanical stirring. Then, a 3 % NaOH solution was added until a white precipitate formed and the pH of the medium exceeded 11. The dispersion was passed through a filter (pore size 0.45 μm), the precipitate was washed with distilled water until neutral pH, placed in 100 ml distilled water, and sonicated using a Qsonica Sonicators (Japan) disintegrator for 10 min (40 kHz). To obtain nanoparticles with ascorbic acid, 50 mg of the latter was added to a solution of chitosan in acetic acid before adding the NaOH solution to it. The other procedures were carried out in the same way as described above.

The associates of nanoparticles with papain were obtained according to the method described in [15] and validated in [16–18].

The protease activity of the obtained complexes was measured as described in [19].

To determine the sizes and surface charges of the papain-nanoparticles associates, we used a Nano Zetasizer ZS (Malvern Instruments, UK) equipped with a 4 mW He/Ne laser with λ = 632.8 nm, the scattering angle was 173°.

In silico studies of the bonds and interactions formed within the chitosan-ascorbic acid-papain complex were performed by flexible molecular docking in the Autodock Vina software (https://sourceforge.net/projects/autodock-vina-1-1-2-64-bit/) using the three-dimensional structure of papain (PDB ID: 9PAP, https://www.rcsb.org/structure/9PAP). The enzyme structure model

was developed and the carrier polymer matrix was optimised as described in [20].

3. Results and discussion

In the first series of experiments, we evaluated the sizes and zeta potentials of chitosan nanoparticle before and after association with papain. The parameters of the papain associates with nanoparticles are presented in Table 1. The median zeta potential of all nanoparticles was 0 mV. It was found that papain associates with medium and high molecular weight chitosan nanoparticles obtained with ascorbic acid differed significantly in size from the ones produced without ascorbic acid. When medium molecular weight chitosan nanoparticles interacted with papain, the size of the associates exceeded the size of free nanoparticles to a greater extent, 42 times for the particles formed without ascorbic acid and 13 times for those obtained with it. On the other hand, for high molecular weight chitosan nanoparticles associated with papain, the size of complexes was only 6 and 8 times larger, respectively, than that of empty nanoparticles. Based on the obtained sizes of papain-chitosan nanoparticles associates, we could assume that the protein adsorption on the surface of nanoparticles was followed by the formation of multilayer structures.

When papain formed complexes with medium and high molecular weight chitosan nanoparticles, obtained without ascorbic acid, the activity of the associates was 94 and 97 % of the values for the native enzyme, respectively. When the papain-nanoparticles complexes with ascorbic acid were formed, the proteolytic activity of the enzyme increased by 3 % for medium molecular weight chitosan and by 16 % for high molecular weight chitosan (Fig. 1). The higher residual papain activity in the complex with chitosan nanoparticles formed with ascorbic acid is probably due to the antioxidant properties of this compound over the biocatalyst [21, 22].

The active site of papain is known to contain a cysteine residue. Its sulphydryl group attacks on the substrate during its hydrolysis. In addition, the SH-group is an effective reducing agent. Therefore, it is easily oxidised by the air oxygen. There are sources reporting on the activation of papain by the introduction of various reducing agents, such as cysteine [23] and other SH-containing compounds [24]. Thus, the fact that papain associates with chitosan nanoparticles and ascorbic acid have higher activity may be attributed to the reducing effect of the acid on the active site SH-group. Moreover, there are reports on the effect of ascorbic acid [25] or its combination with Fe^{2+} [26] or Cu^{2+} [27] ions on the proteolytic activity of native papain. It should also be noted that ascorbic acid has low toxicity and is widely applied for medical purposes. So, it is possible to use enzyme preparations containing it in biomedicine and pharmacy.

We carried out experiments to evaluate the residual activity of papain at 37 °C and pH 7.5

Table 1. Characteristcs of medium and high molecular weight chitosan nanoparticles and papain associates with them

Studied sample	Average size, nm	Size range, nm
Chitosan nanoparticles		
Medium molecular weight chitosan	12	7–21
Medium molecular weight chitosan with ascorbic acid	21	14–59
High molecular weight chitosan	33	18–79
High molecular weight chitosan with ascorbic acid	38	28–79
Papain associates with chitosan nanoparticles		
Medium molecular weight chitosan	499	164–1281
Medium molecular weight chitosan with ascorbic acid	267	91–712
High molecular weight chitosan	200	105–396
High molecular weight chitosan with ascorbic acid	321	105–825

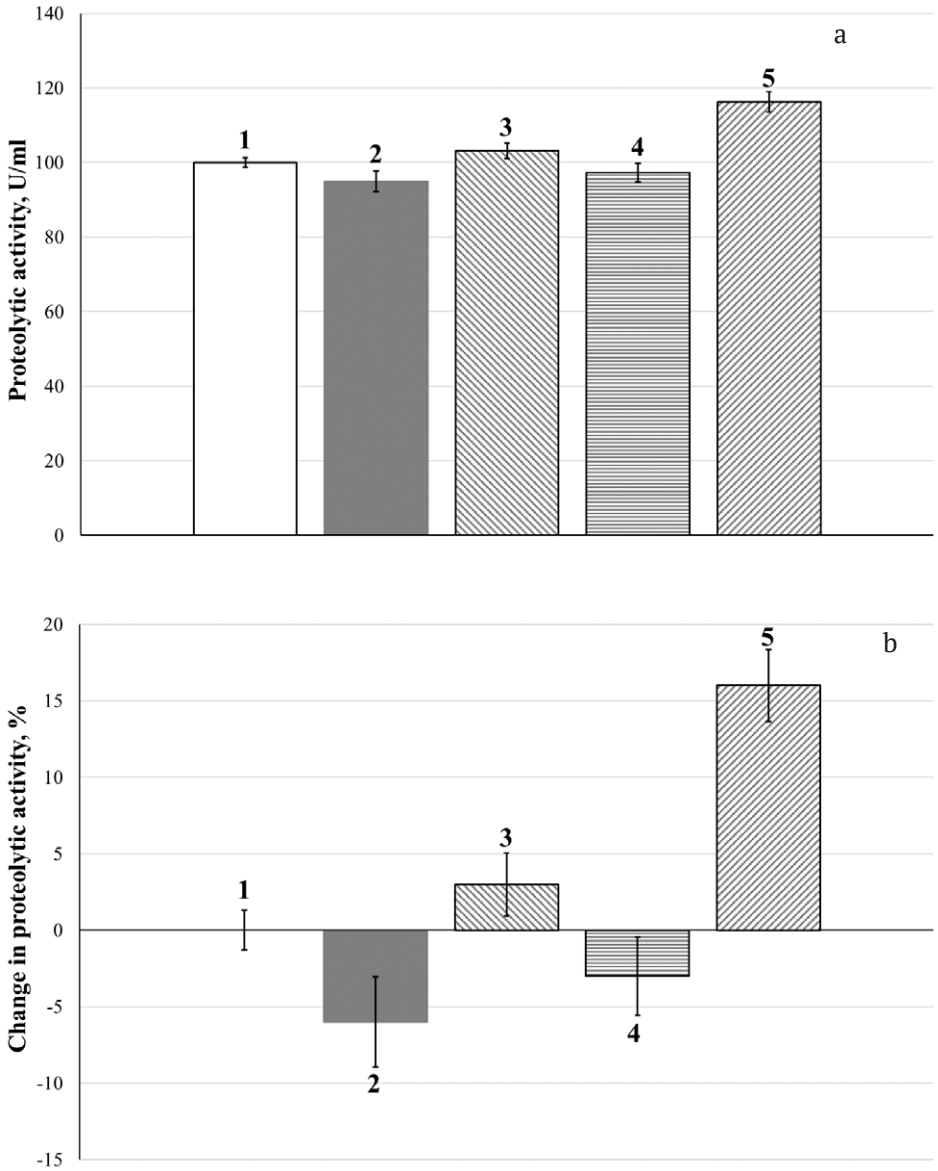

Fig. 1. Catalytic activity of papain, units/ml (A) and its change, % (B): soluble papain (*1*); papain associated with medium molecular weight chitosan nanoparticles (*2*); papain associated with medium molecular weight chitosan nanoparticles with ascorbic acid (*3*); papain associated with high molecular weight chitosan nanoparticles (*4*); and papain associated with high molecular weight chitosan nanoparticles with ascorbic acid (*5*). The activity of free papain under optimum hydrolysis conditions was taken as 100 %

in 0.05 M Tris-HCl buffer for free papain and its associates with medium and high molecular weight chitosan nanoparticles obtained with ascorbic acid and without it. All the samples showed a decrease in their activity within 7 days.

After incubation for 168 hours, native papain solution retained 15 % of its initial proteolytic activity. Its complexes with medium and high molecular weight chitosan nanoparticles, retained, respectively, 29 and 34 % of their ability to hydrolyse azocasein. The associates with medium and high molecular weight chitosan nanoparticles prepared with ascorbic acid kept 40 and 43 %, respectively, of their proteolytic activity (Fig. 2).

Papain associates with both types of chitosan nanoparticles, obtained both with ascorbic acid and without it, were more stable than the free enzyme following 4 hours of incubation in 0.05 M Tris-HCl buffer with pH 7.5 at 37 °C. Thus, complexation with chitosan nanoparticles

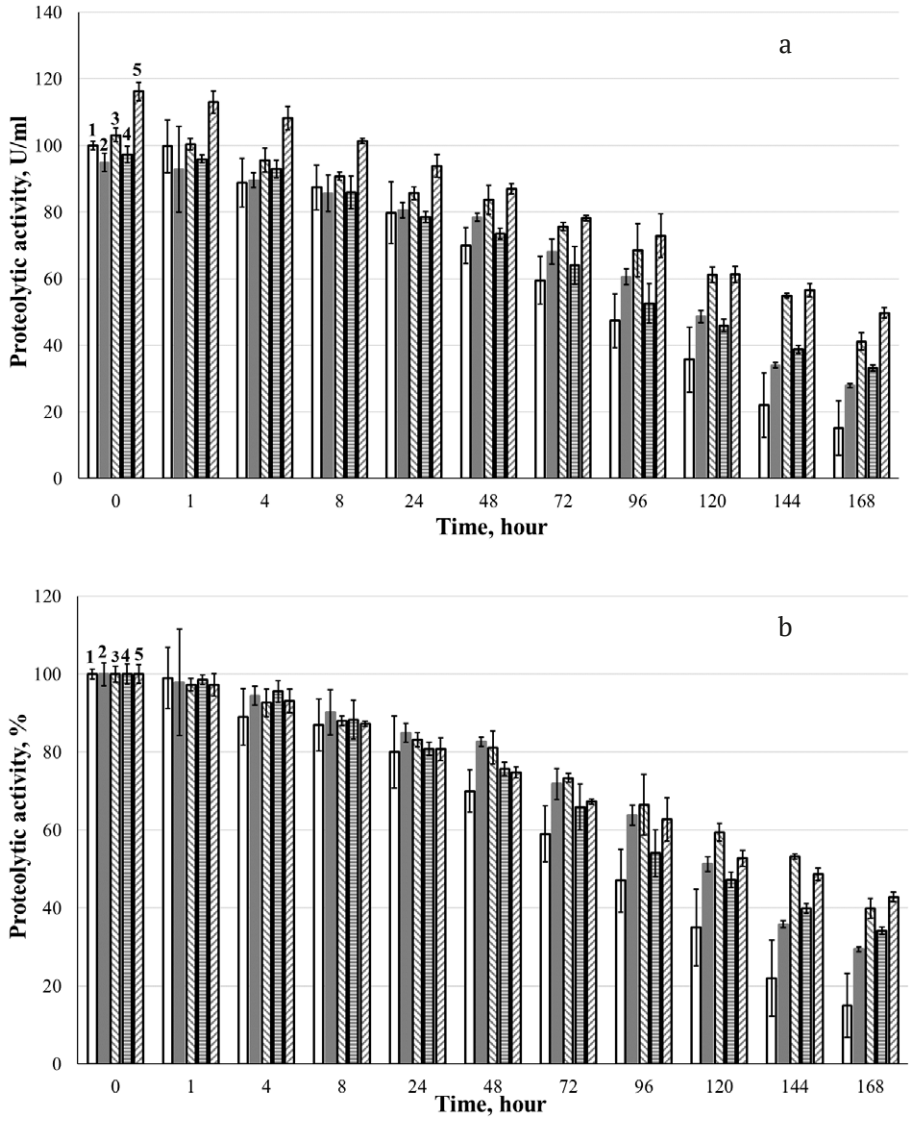

Fig. 2. Residual catalytic activity of papain after incubation of the samples at 37 °C (A: in units/ml of solution or suspension, B: % of the initial value): *1* – soluble papain; *2* – papain associated with medium molecular weight chitosan nanoparticles; *3* – papain associated with medium molecular weight chitosan nanoparticles with ascorbic acid; *4* – papain associated with high molecular weight chitosan nanoparticles; and *5* – papain associated with high molecular weight chitosan nanoparticles with ascorbic acid. The proteolytic activity of the samples without pre-incubation and under optimum hydrolysis conditions was taken as 100 %

increases the stability of papain more effectively than its association with chitosan microparticles under similar conditions. In the case of chitosan microparticles, proteolytic activity stabilisation effects were observed only after 96 hours of incubation in 0.05 M of Tris-HCl buffer with pH 7.5 at 37 °C [16].

To explain the higher residual activity of papain in the complexes with chitosan nanoparticles and ascorbic acid, as well as the increased stability of the enzyme in this complex during incubation at 37°C, we simulated the bonds and interactions within the chitosan-ascorbic acid-papain conjugate (Fig. 3). From the results of an *in silico* study of the interactions within the chitosan-ascorbic acid-papain system it also appears that ascorbic acid does not interact directly with cysteine, which is part of the active site (Cys25). However, it interacts hydrophobically with a catalytically-valuable histidine residue (His159) via the carbon skeleton. This fact indicates

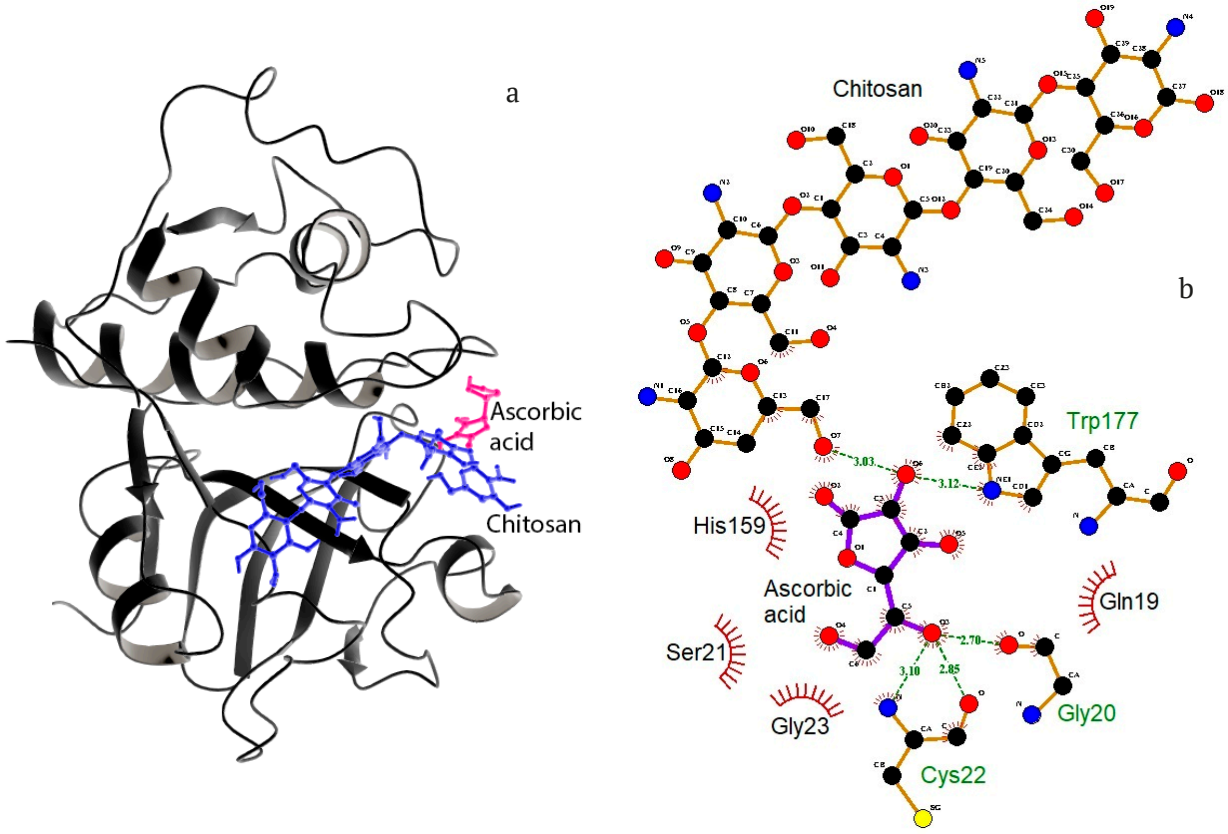

Fig. 3. Topology of the chitosan-ascorbic acid-papain complex (A), and the bonds and interactions between the components of the above system (the dotted lines indicate hydrogen bonds, their lengths are measured in Å (B)

the proximity of the potential reducing agent to the sulphydryl group and also confirms the hypothesis of a reducing effect of ascorbic acid against the active site of papain. Moreover, ascorbic acid has some advantages over other types of reducing agents for cosmetology, biomedicine, and pharmacy: it is a cofactor for a number of enzymes involved in collagen biosynthesis, is necessary for wound healing and bone repair [28], is involved in thyroxine synthesis and amino acid metabolism [21], plays an important role in the antioxidant system, immune competence, and in the resistance to infections [29]. It prevents DNA mutations, and may be an important element in the treatment of some types of cancer and chronic diseases [29]. Chitosan ascorbate is known to have higher antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* than chitosan, which probably inhibits microbial degradation of papain under physiological conditions (37 °C, pH 7.5) [30].

4. Conclusions

Thus, we obtained papain associates with medium and high molecular weight chitosan nanoparticles with and without ascorbic acid. The samples obtained with ascorbic acid exhibited higher proteolytic activity against azocasein.

It was found that papain associates with both types of chitosan nanoparticles and with ascorbic acid significantly differ in size from the associates with particles formed without it. Papain complexes with medium molecular weight chitosan particles exceed the size of free nanoparticles to a greater extent than the enzyme associates with the particles of high molecular weight chitosan.

While determining the stability of the complexes of chitosan and papain nanoparticles, we noticed a decrease in the proteolytic activity of the samples within seven days. The association with chitosan nanoparticles, especially those obtained with ascorbic acid, increased the stability of papain.

To explain the preserved activity of papain in the complexes with chitosan nanoparticles, we analysed *in silico* the interactions within the chitosan-ascorbic acid-papain system. It was determined that ascorbic acid does not bond directly with Cys25 in the papain active site, but involves into hydrophobic interactions with catalytically important amino acid residue His159. This proves the proximity of the potential reducing agent to the sulphydryl group and confirms the hypothesis about the reducing effect of ascorbic acid on the active site of papain.

Contribution of the authors

The authors contributed equally to this article.

Conflict of interests

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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Received 03.10.2022; approved after reviewing 23.11.2022; accepted for publication 15.12.2022; published online 25.06.2023.
Translated by Anastasiia Ananeva
Edited and proofread by Simon Cox