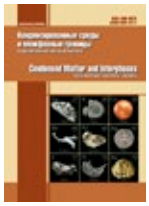

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Biocatalysts based on complexes of carbon nanomaterials with cysteine proteases

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Abstract

The purpose of the research is to develop and study biocatalysts based on complexes of cysteine proteases with fullerenes and carbon nanotubes.

During the formation of ficin complexes with fullerenes and carbon nanotubes, the activity of hybrid preparations was 70 and 45%, respectively. During the formation of papain complexes with fullerenes and carbon nanotubes, the proteolytic ability of the enzyme remained at the same level for the samples with fullerene and decreased by 27% for the preparations with carbon nanotubes. The formation of bromelain complexes with fullerenes and carbon nanotubes contributed to a decrease in the proteolytic activity of the biocatalyst by 18 and 48% as compared to the free enzyme. While determining the stability of complexes of nanomaterials and cysteine proteases during a 7-day incubation in 0.05 M tris-HCl buffer (pH 7.5) at 37 °C, we noticed a decrease in the proteolytic activity of the samples.

Complexation with carbon nanoparticles and fullerenes increased the stability of ficin and bromelain, while the stability of papain in the complexes remained unchanged.

Keywords: Cysteine proteases, Ficin, Papain, Bromelain, Fullerenes, Carbon nanotubes

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1. Introduction

Carbon materials are of great interest to various areas of science. Currently, there is a growing amount of research aimed at expending the application of carbon nanomaterials, including fullerenes and nanotubes. These nanostructures are to some extent considered as possible synthons for biologically active substances [1].

Fullerenes are a new allotropic modification of carbon. The fullerene molecule is a spheroidal hollow framework molecule of an even number of covalently bonded carbon atoms located at the vertices of hexagons or pentagons [2]. Inside the molecule, there is a cavity into which atoms and molecules of other substances can be introduced [3]. It has been established that fullerenes have a stabilising effect on enzymes, which protects them from thermal inactivation and oxidation [4]. In biological systems, fullerenes can have both an antioxidant effect (they add reactive oxygen species) and oxidising effect due to their photosensitising properties. Fullerene molecules are lipophilic and exhibit a membranotropic effect. They interact with various biological structures and can alter their functions, which increases the lipophilicity of the active molecule. Fullerenes can enable the targeted delivery of some therapeutic agents [5], they can be used in X-ray imaging as inhibitors of the process of human immunodeficiency virus multiplication and chemotherapeutic agents. A distinctive feature of fullerenes is their ability to combine several functions, which allows using them in precision medicine. Precision medicine opens a new path to personalised nanomedicine, where the course of the treatment can be controlled and thus adapted for each individual patient [6-8].

Carbon nanotubes are extended cylindrical hollow structures with a diameter of one to several tens of nanometres, a length of tens of microns or, in some cases, of even a centimetre , which are formed by one or more graphene sheets rolled into a seamless tube. Their advantages include a large specific surface area, high stability, strength, thermal conductivity, and unusual electronic and emission properties [5]. The specific surface area of carbon nanotubes is from 150 to 1,500 $m^2 \cdot g^{-1}$, which is many times higher than that of fullerenes [9, 10]. Carbon nanotubes have the potential to be used as safe and effective alternatives to existing drug delivery methods: they can pass through the membranes together with treatment medications, vaccines, and nucleic acids and penetrate deep into the cell to reach substrate targets; they serve as ideal non-toxic carriers, which in some cases increase the solubility of the preparation and enhance its efficiency and safety [11]. Carbon nanotubes were used to develop estrogen and progesterone test strips, DNA and protein microarrays, and NO_2 and cardiac troponin sensors. Similar sensors have been used to detect gases and toxins [12-14].

A high specificity of enzyme catalysis provides for an impressive target product yield and an almost waste-free production. Proteolytic plant enzymes are often used in medicine. The most popular among them are ficin (EC 3.4.22.3), bromelain (EC 3.4.22.32), and papain (EC 3.4.22.2) [15,16].

Ficin (EC 3.4.22.3) is made from the *Ficus* plants. It is a cysteine proteolytic enzyme. The molecular weights of the enzyme is 25-26 kDa. Ficin has a wide range of pH values (6.5-9.5) in which it exhibits high activity [17]. The isoelectric point of the enzyme is 9.0. The ficin molecule consists of a single polypeptide chain with an N-terminal leucine residue [18-20]. Ficin exhibits antimicrobial activity against gram-positive and gram-negative bacteria. In addition, it is also known to have anti-inflammatory, anthelminthic, antithrombotic, fibrinolytic, and anti-cancer properties and an immunomodulatory effect [21, 22].

Papain (EC 3.4.22.2) is extracted from papaya (*Carica papaya*). Its molecular weight is 23 kDa. The enzyme consists of 212 amino acid residues with isoleucine at the N-terminus and asparagine at the C-terminus. Papain has a high activity in different media: at pH 5.0-7.5, it hydrolyses proteins, peptides, and amides. The most favourable temperature for the enzyme functioning is in the range of 50–60 °C. Its isoelectric point is 8.75 [23–25]. Papain can break down proteins with a greater speed and efficiency than many animal and bacterial enzymes, it can contribute to a faster healing of wounds, bedsores, and trophic ulcers, it has anti-inflammatory properties, and allows other drugs to penetrate the skin without violating its integrity [26–29].

Bromelain (EC 3.4.22.32) is a proteolytic plant enzyme which is made from pineapples. The molecular weight of bromelain is 33 kDa and the isoelectric point is 9.55. The most favourable temperature for the enzyme is 62 °C, and the pH is 7.0 [30, 31]. Bromelain is used to improve digestion, to mitigate the symptoms of inflammatory processes, to reduce edema, and to increase the rate of tissue regeneration. It is characterised by anti-cancer properties, it can prevent thrombus formation, accelerates tissue regeneration processes during depolymerisation of intercellular structures, changes the permeability of blood vessels, and has an immunomodulatory effect [32–35].

However, there are several reasons that prevent the large-scale use of the enzymes: the instability of the preparations under various conditions, high cost, and impossibility of their repeated use. These problems can be largely overcome by using associated enzymes, which are more stable and have a sustained action [36, 37].

Therefore, the aim of this research was to develop biocatalysts based on complexes of cysteine proteases with carbon nanotubes and fullerenes and to study their catalytic activity.

2. Experimental

Ficin, papain, and bromelain were in the focus of the study and azocasein (Sigma, USA) was used as a substrate for hydrolysis. For complexation, the following certified carbon nanomaterials were used: Nanocyl-7000 nanotubes (NANOCYL S.A.) with a length of 0.7–3.0 μm and a diameter of 5–35 nm; C_{60} NeoTechProduct fullerenes with a purity of 99.5%.

The enzyme complex with carbon nanotubes and fullerenes was prepared as follows: an enzyme solution (2 mg/ml in 50 mM of glycine buffer, pH 10.0 and 9.0 for ficin and papain and in 50 mM of tris-glycine buffer, pH 9.0 for bromelain) was mixed in equal volumes with a solution of carbon nanotubes and fullerenes and kept at room temperature for 2 h. The protease activity of the obtained compounds was measured as described in [38].

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

3. Results and discussion

In the first series of experiments, we determined the size and zeta potential of fullerenes and carbon nanotubes. The parameters of the nanoparticles are presented in Table 1. The average size of fullerenes was 113 nm and the average size of carbon nanotubes was 153 nm. The median zeta potential was –12 mV for fullerenes and –20 mV for carbon nanotubes.

During the formation of ficin complexes with fullerenes and carbon nanotubes, the activity of the associates was 70 and 45% of the values for the native enzyme, respectively. During the formation of papain complexes with fullerenes and carbon nanotubes, the proteolytic ability of the enzyme remained at the same level for the fullerene and decreased by 27% for the carbon nanotubes. The formation of bromelain complexes with fullerenes and carbon nanotubes contributed to an 18 and 48% decrease in proteolytic activity as compared to the free enzyme (Fig. 1).

We conducted experiments aimed to determine the residual activity of cysteine proteases at 37 °C and pH 7.5 in 0.05 M HCl buffer for free enzymes and their complexes with fullerenes and carbon nanotubes. All samples showed a decrease in their activity within 7 days.

A solution of native ficin after a 7 day incubation retained 8% of its original proteolytic activity, its complexes with fullerenes and carbon nanotubes showed 46 and 43% of their ability to hydrolyse azocasein, respectively. Native papain after its incubation for 7 days retained 15% of its activity, and papain samples with fullerenes

Table. 1. Parameters of nanoparticles

Nanoparticles	Average size, nm	Size range, nm	Median zeta-potential, mV	Zeta-potential range, mV
Fullerenes	113.8	91.2-141.8	–12.3	from –25.8 to 13.9
Carbon nanotubes	153.4	122.4-190.1	–20.1	from –35.7 to –5.99

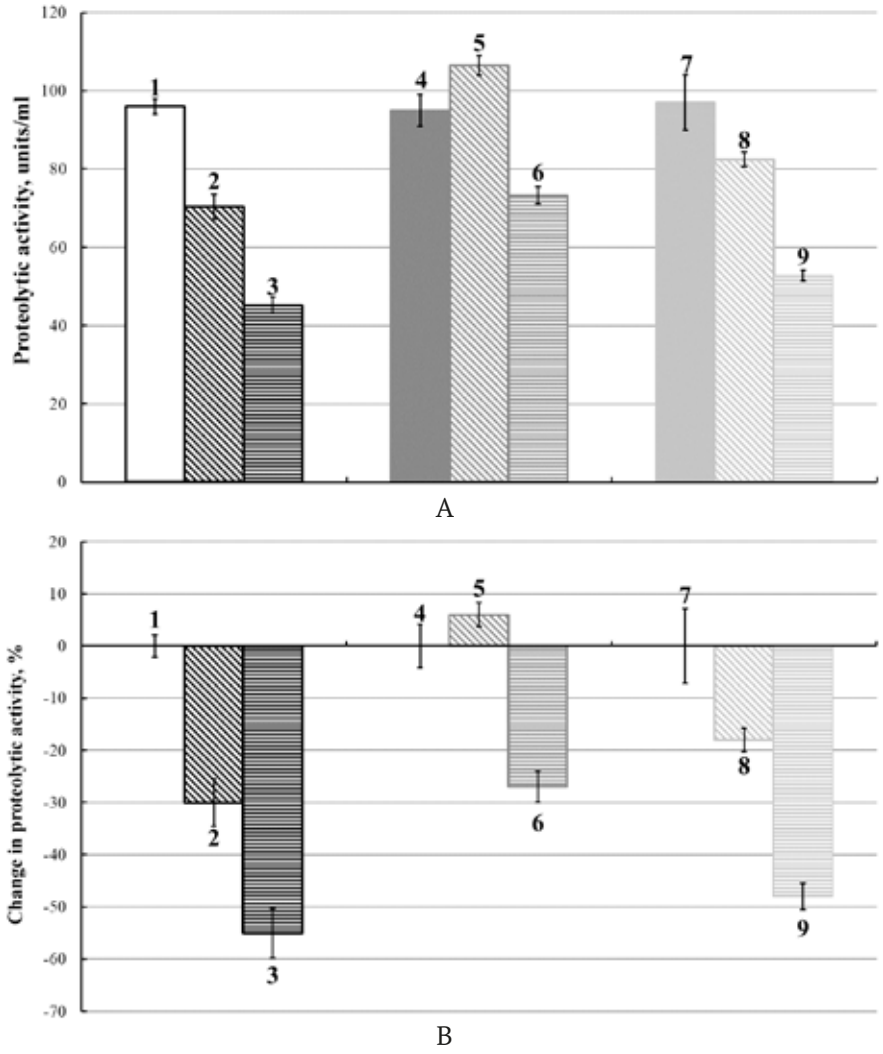

Fig. 1. Catalytic activity of ficin, papain, and bromelain, units/ml (A) and its change, % (B): soluble ficin (1); ficin complex with fullerene (2); ficin complex with carbon nanotubes (3); soluble papain (4); papain complex with fullerene (5); papain complex with carbon nanotubes (6); soluble bromelain (7); bromelain complex with fullerene (8); bromelain complex with carbon nanotubes (9). The activity of free enzymes under optimum hydrolysis conditions was taken as 100%

and carbon nanotubes showed 27 and 22%. The bromelain solution retained 13% of its proteolytic activity after 7 days of incubation, while its complexes with fullerenes and carbon nanotubes retained 26 and 29% (Fig. 2).

4. Conclusions

Therefore, as a result of research we obtained complexes of cysteine proteases with fullerenes and carbon nanotubes. Papain complexes with fullerenes showed higher values of proteolytic activity in relation to azocasein than the other studied biocatalysts.

While determining the stability of the complexes of nanoparticles and cysteine proteases, we noticed a decrease in the proteolytic activity of the samples within seven days. Both complexation with fullerenes and carbon nanotubes increased the stability of ficin and bromelain, while the stability of papain in the complexes remained at the level of the free enzyme.

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.

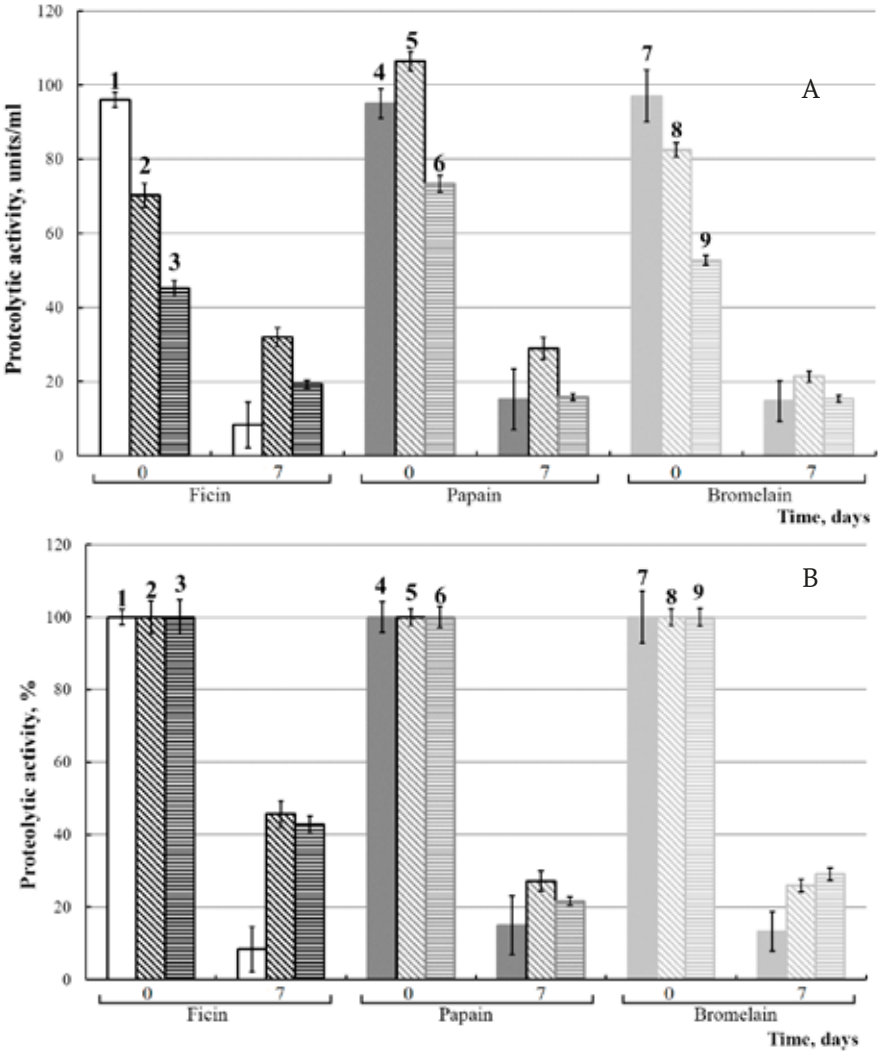

Fig. 2. Residual catalytic activity of ficin, papain, and bromelain after the incubation of samples at 37 °C ((A) in units/ml of solution or suspension, (B) in % of the initial level): soluble ficin (1); ficin complex with fullerene (2); ficin complex with carbon nanotubes (3); soluble papain (4); papain complex with fullerene (5); papain complex with carbon nanotubes (6); soluble bromelain (7); bromelain complex with fullerene (8); bromelain complex with carbon nanotubes (9). The proteolytic activity of the samples without pre-incubation and under optimum hydrolysis conditions was taken as 100%

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