Volume 8, Issue 3, 2018, 3224 - 3231 ISSN 2069-5837
Biointerface Research in Applied Chemistry
www.BiointerfaceResearch.com
Original Research Article Open Access Journal
Received: 22.04.2018 / Revised: 28.05.2018 / Accepted: 10.06.2018 / Published on-line: 15.06.2018

Biodegradable poly-3-hydroxybutyrate as a shielding carrier for a plant-protecting MF3 protein from *Pseudomonas fluorescens*

Sofya Popletaeva[1], Tatiana Voinova[1], Maksim Kartashov[1], Tatiana Pasechnik[1], Lenara Arslanova[1], Larisa Shcherbakova[1], Natalia Statsyuk[1,*], Anton Bonartsev[2], Anton Zernov[2], Garina Bonartseva[2], Vitaly Dzhavakhiya[1]

[1]All-Russian Research Institute of Phytopathology, Bolshie Vyazemy, Moscow region, 143050, Russia
[2]Federal Research Centre "Fundamentals of Biotechnology", Russian Academy of Sciences, Moscow, 119071, Russia

*corresponding author e-mail address:***nataafg@gmail.com***

ABSTRACT

Boosting of plant immunity to improve its resistance to pathogens is a promising approach to increase crop capacity and reduce pesticide press on the environment. A typical scenario of such approach is based on the use of elicitors able to activate key plant defense mechanisms. MF3, a highly thermostable FKBP-type peptidyl prolyl cis/trans isomerase from *Pseudomonas fluorescens*, has a proved eliciting activity regarding various crops. However, for field application, this enzyme should be protected against degrading action of sunlight, as well as plant and microbial proteinases. In this study we examined a possibility to encapsulate MF3 using biodegradable poly-3-hydroxybutyrate (PHB) and evaluated the efficacy of the resulted MF3 shielding against UV radiation and enzymatic proteolysis, as well as the resistance-eliciting activity of the encapsulated protein. PHB-based microparticles loaded with MF3 in a complex with low-molecular chitosan and 500-kDa dextran were obtained using a Solid/Oil/Water technique followed with a spray drying and freeze frying. The resulting microparticles consisted of PHB (80%), chitosan (4%), dextran (6%), and MF3 (10%) and had a size of 10-25 μm. The effective loading capacity was ~10%; *in vitro* kinetic study showed that 89% of the total loaded protein was released within the first 24 h, while the rest of the protein slowly released within next 12 days. A comparison of the resistance-eliciting activity of free and encapsulated MF3 was carried out using two model "plant-pathogen" systems, tobacco/tobacco mosaic virus (TMV) and tobacco/*Alternaria longipes*. In both models, activity of encapsulated MF3 was similar to that of free MF3 of the same concentration and resulted in the reduction of infection level by 30.3–36.8% for *A. longipes* and 66.8–68.4% for TMV. *In vitro* assessment of the effect of UV irradiation and proteinase K treatment on the activity of free and encapsulated MF3 showed a clear shielding effect of PHB. Even after 8 h of UV treatment, when free MF3 completely lost its resistance-eliciting activity, encapsulated MF3 still provided almost 50% suppression of TMV infection. Treatment with proteinase K (20 and 100 μg/mL) resulted in significant loss of free MF3 protecting efficiency (22.9 and 35.5%, respectively), while for encapsulated MF3 this loss made only 2.6 and 12.2%, respectively. These results confirmed the encapsulation procedure did not impair the protective activity of MF3, while provided a successful release of this protein from the complex and its delivery to putative receptors of plant cells. The study has shown a principal possibility to produce PHB-based MF3 preparations characterized by improved resistance of their bioactive component to adverse biotic and abiotic factors and possessed a significant protective activity in relation to the tested plant pathogens. The further improvement of the encapsulation technology and the characterization of encapsulated MF3 using other model systems and whole plants are planned.

***Keywords*:** *poly-3-hydroxybutyrate; MF3; FKBP-type peptidyl prolyl cis/trans isomerase; protein elicitors; encapsulation; plant resistance inductors; tobacco mosaic virus; Alternaria longipes; UV protection.*

1. INTRODUCTION

Agricultural crops are under constant threat of an attack of various plant pathogens able to significantly reduce their productivity. To resist them, plants contain a sophisticated immune system, which, in addition to passive barriers (plant cell wall, cuticle, etc.), provides a range of active responses to various molecules of plant or pathogen origin, which are released during a plant–pathogen interaction. Nevertheless, plant immune system is not able to completely prevent a harm caused by plant pathogens. According to some data, in the case of the five most important crops (wheat, rice, maize, potato, and soybean), potential global losses caused by various microbial pathogens and viruses may reach 12.3–29.3% depending on the crop; however, various crop protection techniques are able to reduce these losses [1]. Among the protection methods, treatment with chemical pesticides plays a major role. The volume of seasonal treatments of crops is often significant. For example, in some European countries, potato fields are sprayed with 5- to 7-day intervals [2]. Such intensive use of chemicals may cause accumulation of pesticide residues in soil and plants resulting in various environmental pollution and food safety issues. In addition, active and regular use of pesticides often results in the emergence of resistant strains of pathogens that causes the application of higher dosages of chemicals to overcome their resistance. In recent years, reduction of the pesticide use became a subject of a global concern, and governments of many countries are searching for any environmentally friendly alternatives for crop protection.

One of the possible solutions is a development of tools to boost plant immunity and to improve their own resistance to

pathogens. The most common scenario of this approach is based on a so-called induced resistance representing activation of key plant defense mechanisms by compounds mimicking pathogen attacks. Such compounds called general elicitors are recognized by theinnate immune system and prime or induce defense responses including systemic ones [3, 4]. They are effective against a wide range of plant pathogens (viruses, bacteria, fungi, oomycetes, and nematodes). The mode of their action differs from traditional chemical pesticides because they do not directly target a pathogen, but rather indirectly inhibit disease development via the triggering of plant defense responses. As a result, pathogens do not develop resistance to elicitors [5, 6]. Thus, the use of elicitors may be a good and promising alternative to chemical pesticides.

Elicitors may be chemically synthesized or biological in origin; in the last case, they may represent plant-derived or microbial compounds [4]. Microbial elicitors may include cell wall fragments, glycoproteins, lipids, poly- and oligosaccharides, organic acids, and also various peptides and proteins. The last group includes elongation factor Tu, harpins, flagellin, elicitins, cold shock proteins, and some other proteins and enzymes [7–11]. Since peptides and proteins specifically interact with target receptors, they may possess a higher protecting efficiency than other organic compounds [12]. In recent years, searching for novel protein elicitors has become popular among scientists working in the field of a plant disease control [13–19]. Some of the revealed protein elicitors have been already commercialized as biopreparations. Among them, one can mention the Messenger®, a harpin-based biopreparation registered by Eden Biotechnology Co. for use on all crops [20]. It was shown to be quite efficient on a range of crops, such as tomato, grape, muskmelon, etc. [21–23], though some contradictory data regarding its efficiency and range of action have been also published [24, 25]. Another known commercial biopreparation, ATaiLing, is based on a PeaT1 protein produced by *Alternaria alternata* and showed significant antiviral effect [26]. In addition, the product contains chitosan-based oligosaccharides. In 2015 it was awarded as the top-selling crop protection product in China. To date, ATaiLing production reached 800 tons/year [20]. In our earlier studies, we revealed several proteins able to protect plants against a range of pathogens. One of such proteins called MF3 was isolated from *Pseudomonas fluorescens* and identified as a FKBP-typepeptidyl prolyl cis/trans isomerase (PPIase); this protein provides protection of various crops, such as wheat, rice, tobacco, potato, and cabbage via induction of their resistance to several viruses and fungi [27, 28]. The effect was observed for both applications of MF3 preparations on the surface of seed and tubers, or spraying of vegetative plants. In the further studies, we determined a primary structure of MF3 and its active center [29]. Being highly thermostable and produced by an overexpressing recombinant *Escherichia coli* BL21 (DE3) strain, MF3 seems to be very promising for practical use.

At the same time, development and application of protein elicitors for crop protection should take into account some concerns related to their decreased efficiency under field conditions as compared with laboratory or greenhouse trials. This variation in efficiency may range from between 20–85% and depends on some biotic and abiotic factors, such as UV radiation, temperature, and proteinases of microbial and plant origin able to degrade protein molecules [30]. Elimination of these threats via protection and stabilization of protein molecules would provide better efficiency of protein-based preparations resulting in a lower effective concentration and prolonged action. A possible solution is the use of protein elicitors in a combination with biopolymers able to shield them against adverse external factors and to facilitate their interaction with plant cell receptors while keeping their biological activity. Though complexation of proteins with biopolymers is commonly used in medicine for protection and delivery of various drugs [31], implementation of this approach in agriculture is limited mainly by shielding of beneficial soil bacteria and provision of a controlled release of chemical pesticides and fertilizers [32, 33]. Therefore, the problem of development of “protein elicitor–biopolymer” complexes with improved resistance to adverse external factors still remains poorly studied, so the development of such stabilized complexes requires thorough selection and assessment of a biopolymer to be used.

The purposes of this study were the construction of a MF3 complex with poly-3-hydroxybutyrate widely applied in biology and medicine [34], the assessment of a shielding efficacy of this biopolymer in relation to UV radiation and enzymatic proteolysis, and the comparison of a plant-protecting activity of the obtained complex and intact MF3 using two model “plant-pathogen” systems.

2. EXPERIMENTAL SECTION

Materials. MF3 and PHB were obtained by a microbiological synthesis (see below). Other chemicals used in the study were purchased in Sigma-Aldrich excepting polyvinyl alcohol (MP Biomedicals, USA) and chloroform, acetic acid, and dichloromethane (Ekos-1, Russia).

MF3 production and isolation. MF3 was produced using a recombinant overproducing *E. coli* BL21 (DE3) strain [35]. Strain cultivation and MF3 isolation were carried out according to the earlier described procedures [29].

MF3 purification. The purification of the isolated MF3 was carried out according to the earlier described procedures [36].

PHB production and purification. Low-molecular PHB used for the preparation of biopolymer microspheres was obtained using an overexpressing *Azotobacter chroococcum* 7B strain and purified as described earlier [37].

Obtaining of PHB-based complexes. MF3 incorporation into a polymer PHB matrix was carried out using a Solid/Oil/Water technique [38]. Chitosan and dextran were used as additional protein-stabilizing components.

First, complexes of MF3 with low-molecular chitosan and 500-kDa dextran (MF3/CHI/DEX) were prepared by a spray drying method [39]. The following solutions were prepared: 10% chitosan solution in 1% acetic acid, 10% water solution of dextran and water solution of MF3 (15 mg/mL, 10 mL). Each solution was filtered through a G3 glass filter (Schott Duran, Germany). To obtain a working solution, chitosan and dextran solutions were added to a protein solution and thoroughly mixed so that the final content of these three components was 150 mg of MF3, 60 mg of chitosan, and 90 mg of dextran. Then the solution was supplemented with distilled water up to a final volume of 50 mL and loaded on a Buchi Nano Spray Dryer B-90 atomizing drier (Buchi, Switzerland). The resulting microparticles were collected by a special spatula (Buchi electrostatic particle collector) and vacuumed overnight on an ALPHA 1-2 LD plus freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Germany) to remove residual water. The product yield (%) of a MF3/CHI/DEX complex was calculated as a ratio of the total weight of microparticles to the sum of weights of their components used in the reaction.

To cover the obtained complexes with PHB, MF3-loaded microparticles and protein-free microparticles (control), produced at the previous stage, were suspended in 2 mL of PHB solved in dichloromethane (30 mg/mL). The mass ratio of PHB to any of these two variants of microparticles was 4:1. The obtained colloid solution was emulsified via gradual addition to 100 mL of 1.5% polyvinyl alcohol in distilled water (w/v) at 40ºC under constant stirring (1000 rpm, 2 h) on a RZR 2021 mechanic stirrer (Heidolph, Germany). After complete evaporation of the organic solvent, the obtained MF3/CHI/DEX@PHB microspheres were separated by centrifugation (4400 rpm, 6 min) using a 5702 R centrifuge (Eppendorf, Germany), then thrice washed with distilled water to remove polyvinyl alcohol from their surface, and freeze-dried overnight using an ALPHA 1-2 LD plus freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Germany).

To determine the effective loading capacity (LC, %), a sample of emulsifier (polyvinyl alcohol) was taken at the end of the emulsification process, and the content of non-incorporated MF3 was determined spectrophotometrically at 280 nm using a UV-1601PC spectrophotometer (Shimadzu, Japan). The LC value was calculated using the following formula:

$$LC = \frac{\mathrm{MF3}_{total} - \mathrm{MF3}_{em}}{M} \cdot 100\,\%,$$

where $MF3_{total}$ is the total weight of the protein used, $MF3_{em}$ is the weight of MF3 containing in emulsifier, and M is the total weight of microparticles.

Evaluation of the size of PHB-based microparticles and kinetics of MF3 release. The morphology and size of the obtained microspheres were examined using a JEOL JSM-638 OLA scanning electron microscope (FEI Company, USA); prior examination, samples were coated with palladium.

The ability of PHB-based microspheres to release the loaded protein was determined *in vitro*. MF3-loaded microparticles were suspended in 1 mL of 0.1 M phosphate buffer (pH 7.2) containing sodium azide (0.02% w/v) as an antimicrobial agent up to a final concentration of 20 mg/mL. Microparticles were incubated for 10 days at 25–27ºC under constant shaking at 330 rpm using a TS 1/20 thermostat (Smolenskoe SKTB SPU Ltd., Russia) and an OS-10 orbital shaker (Biosan, Latvia). During the experiment, every 1.5 h for the first 24 h and then every 24 h until the end of the experiment, microspheres were centrifuged (10000 rpm, 10 min) using a MiniSpin centrifuge (Eppendorf, Germany), the supernatant was taken into a new tube, and the sedimented microspheres were supplemented with a fresh portion of the buffer to continue incubation. The protein concentration in the supernatant was determined spectrophotometrically at 280 nm using a UV-1601PC spectrophotometer (Shimadzu, Japan). Results read versus phosphate buffer were compared with the calibration curve generated for MF3 dissolved in phosphate buffer at different concentrations.

Plants and pathogens. Protective activity of the studied microparticles was evaluated in model plant–pathogen systems using tobacco (*Nicotiana tabacum*) plants and two pathogens of different nature, tobacco mosaic virus (TMV) and *Alternaria longipes* fungus causing tobacco brown rot.

Tobacco seeds (necrosis-forming cultivar Xanthi NN) were sown into vegetation pots filled with soil. After shoot appearance, plants were grown for two weeks, then transplanted into individual 400-mL pots, and continued to grow in a climatic chamber at a 16-h photoperiod and 22°C/20°C (day/night) temperature up to the stage of 3–4 true leaves. All experiments were performed using detached mid-layer leaves.

The LS-1 strain of TMV and the strain 100055 of *A. longipes* were provided by the State Collection of Phytopathogenic Microorganisms of the All-Russian Research Institute of Phytopathology (ARRIP).

The LS-1 strain was maintained on tobacco plants (cv. Samsun). LS-1-inoculated plants were grown in a climatic chamber at a 16-h photoperiod and 24°C/20°C (day/night) temperature.New plants were inoculated as required by rubbing of their leaves, preliminarily sprayed with carborundum powder, with a homogenate of TMV-infected tobacco leaves [7]. To assess the protective activity of the MF3 and MF3/CHI/DEX@PHB complex against TMV, the homogenate was diluted prior inoculation so that the applying of its 50-60 μL aliquote upon a surface of a leaf half resulted in a formation of 50–100 necroses.

A. longipes strain 100055 was grown in sterile Petri plates with potato glucose agar for 2–3 days at 22ºC. Then plates were incubated for 7–8 days in a chamber with UV background illumination to initiate spore formation. The spore suspension was obtained by the washing of Petri plates with sterile 50 mM glucose and adjusted to a working concentration (10^5 spores/mL).

Assessment of the protecting activity of free and encapsulated MF3 against TMV and *A. longipes*. Left halves of detached tobacco leaves were sprayed with MF3 (1 μg/mL), MF3/CHI/DEX@PHB (10 μg/mL, i.e., 1 μg/mL in relation to MF3), and CHI/DEX@PHB preparations (10 μg/mL), whereas right halves were treated with distilled water (control). After a 24-

h incubation in a wet chamber at room temperature, leaves were inoculated with TMV as described above or sprayed with *A. longipes* spore suspension [40] and returned to the wet chamber. After 3–4 days of incubation, the number of appeared necroses was calculated for each leaf half. The infection index (%) was calculated as a ratio between the number of necroses on the experimental and control leaf halves. Each experiment was carried out in five replications.

Photostability assay. Free or encapsulated MF3 was dissolved in a 200 μL of sterile distilled water up to a final concentration of 1 and 10 μg/mL, respectively, and placed onto a surface of the watch glass. The glasses were placed into a wet chamber and UV-treated using a Mineral Light G-80 UV lamp (36 W, 210–320 nm) placed at a distance of 43 cm. The treatment was carried out for 8 h at room temperature; within this period, no significant water evaporation from the solutions was observed. During the treatment, aliquots of both preparations were sampled at a certain time interval to evaluate their protecting activity using a "tobacco/TMV" model system as described above. The experiment was carried out in four replications.

Assessment of the shielding activity of PHB against enzymatic proteolysis. The experiment was performed using detached tobacco leaves. The left half of each leaf (control) was sprayed, depending on the experimental variant, with a water solution of proteinase K in three different concentrations (0, 20, and 100 μg/mL). The right half (experiment) was sprayed with the mix of proteinase K in the corresponding concentration and the MF3 (1 μg/mL) or MF3/CHI/DEX@PHB (10 μg/mL) preparation. Treated leaves were placed into a wet chamber and incubated 24 h at room temperature, then inoculated with TMV as described above and left in a wet chamber for five days. At the end of incubation, the number of necroses per each leaf half was counted and the infection index (%) was calculated for each leaf as described above. The experiment was carried out in four replications.

Data treatment. The data were statistically treated using a MS STATISTICA 6.0 software (StatSoft Inc., USA). The reliability of differences between the control and experimental values was evaluated using an Independent Sample T Test ($p < 0.05$). Data presented on diagrams are shown as M ± SE, where M is a mean value, and SE is a standard error.

3. RESULTS AND DISCUSSION SECTION

Obtaining of a MF3/CHI/DEX@PHB complex. An attempt of a direct MF3 incorporation to PHB showed that the resulting complex was unstable; during emulsification, MF3 was dissociated from PHB and dissolved in the aqueous solution of the emulsifier (polyvinyl alcohol). To solve this problem, we modified the initial technology. Prior encapsulation in PHB, MF3 was mixed with chitosan, a polycation, which is insoluble in water at neutral pH values. In addition, the choice of this carrier for our study was influenced by its proved eliciting activity [41]. To overcome an increased viscosity of this polyelectrolyte, which was undesirable for the further spray drying process, an additional neutral polymer (500-kDa dextran) was also added. At the stage of the preparation of MF3/CHI/DEX particles, the yield of the resulting carrier complex was 68%. After encapsulation of this complex in PHB, we obtained microparticles of the following composition: PHB (80%), chitosan (4%), dextran (6%), and MF3 (10%).

The electron microscopy study of MF3/CHI/DEX@PHB microparticles showed they have a spherical form and quite smooth surface with the size varying within 10–25 μm (Figure 1).

Kinetic study of a protein release from the MF3/CHI/DEX@PHB complex. To confirm a sufficient permeability of the obtained microspheres for proteins, an *in vitro* study of the protein release kinetics was performed (data not shown). The most intensive protein release (89% of the total released protein) was observed within the first 24 h; then the process delayed, and the rest of the protein slowly released within 12 days. The calculated effective loading capacity (LC) of PHB-based microspheres was slightly less than 10%.

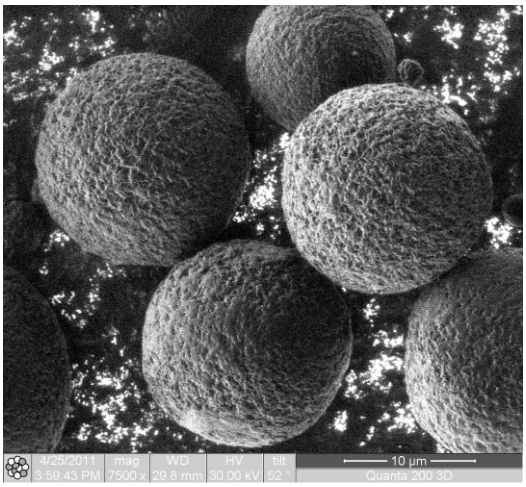

Figure 1. Morphology of PHB-based microparticles loaded with MF3.

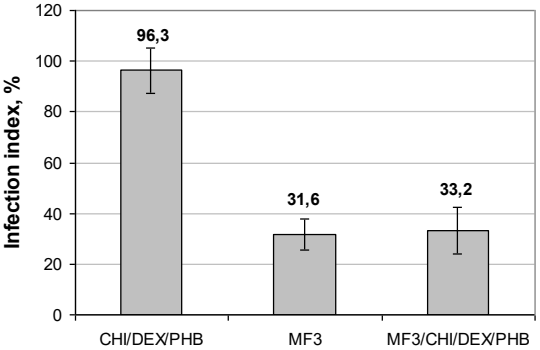

Figure 2. Protective effect of free and encapsulated MF3 and MF3-free microparticles in relation to tobacco leaves (cv. Xanthy NN) infected with tobacco mosaic virus. The least significant difference ($LSD_{0.95}$) is 25.2.

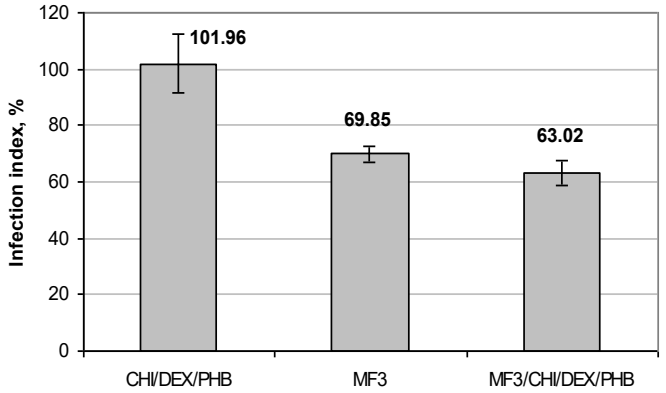

Figure 3. Protective effect of free and encapsulated MF3 and MF3-free microparticles in relation to tobacco leaves (cv. Xanthy NN) infected with *Alternaria longipes* ($LSD_{0.95}$ = 21.4).

Comparison of a protecting activity of MF3 and MF3/CHI/DEX@PHB complex. Since encapsulation of protein-based drugs using various biopolymers may result in the loss of their activity [42], the protection effect of MF3 and MF3/CHI/DEX@PHB complex was *in vitro* compared using two model "plant–pathogen" systems (tobacco/TMV and tobacco/*A. longipes*). In addition, this effect was determined for MF3-free CHI/DEX/HPB complex. Results of the experiments are shown in Figures 2 and 3.

In both cases, no significant protecting activity for MF3-free microparticles was obtained, i.e., CHI/DEX@PHB complex does not induce resistance in the case of the two pathogens used. At the same time, treatment of leaves with free and encapsulated MF3 provided a significant reduction of the infection level for both TMV and *A. longipes* (66.8–68.4 and 30.3–36.8%, respectively). Since no significant difference was observed between the effects of free and encapsulated MF3, the protein did not lose its activity after encapsulation.

Assessment of a shielding activity of PHB against UV irradiation and enzymatic proteolysis. Results of assessment of the protecting activity of UV-treated MF3 and MF3/CHI/DEX@PHB complex using a "tobacco/TMV" model system are shown in Figure 4. For all UV treatment variants, application of encapsulated MF3 resulted in a lower infection index comparing to free MF3. After 8 h of UV treatment, free MF3 completely lost its activity (infection index increased to ~100%). In contrast, encapsulated protein still provided a twice lower infection index. Therefore, encapsulation in PHB provides a significant UV-shielding of MF3.

Results of assessment of the protecting activity of MF3 and MF3/CHI/DEX@PHB complex treated with proteinase K are shown in Figure 5.

Proteinase K itself did not provide any effect on the number of developed necroses. Treatment with proteinase K reduced protection efficiency of both studied preparations; the higher the proteinase K concentration, the more pronounced the effect. At the same time, encapsulated MF3 conferred significantly higher resistance to TMV than free MF3. Treatment of tobacco leaves with the protein preparations preliminarily exposed to 20 and 100 μg/mL proteinase K solutions increased the infection index by 22.9 and 35.5% for MF3 and only by 2.6 and 12.2% for the MF3/CHI/DEX@PHB complex, respectively, as compared to the variants without proteinase K application.

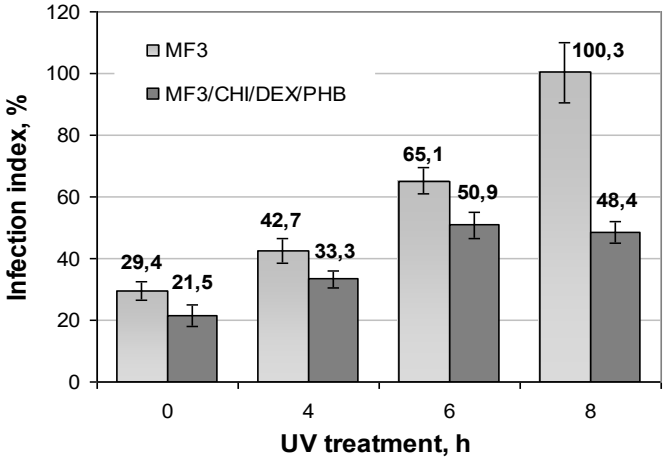

Figure 4. Effect of UV irradiation on the protecting activity of MF3 and MF3/CHI/DEX@PHB complex against tobacco mosaic virus.

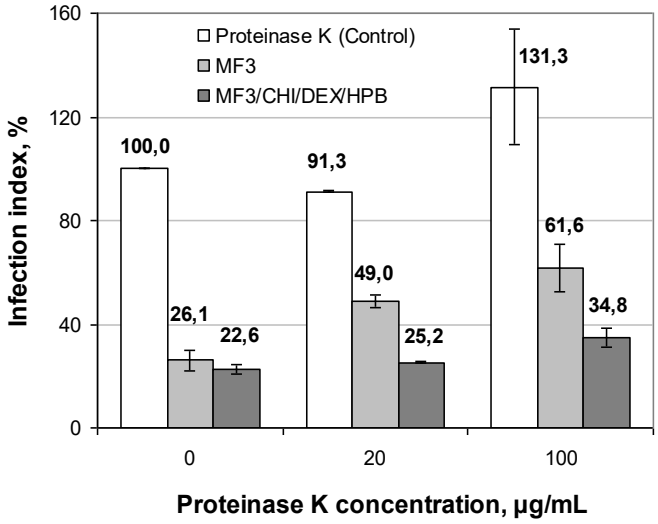

Figure 5. Effect of proteinase K treatment on the protecting activity of MF3 and MF3/CHI/DEX@PHB complex against tobacco mosaic virus. White bars indicate control (treatment with a water solution of proteinase K of the corresponding concentration), grey and dark-grey bars indicate experimental variants (MF3 or MF3/CHI/DEX@PHB, respectively) treated with a proteinase K solution of the corresponding concentration.

Today the development and study of biodegradable polymer systems for encapsulation of biologically active substances and provision of their controlled release is one of the most relevant problems of the modern biotechnology. The use of such systems allows researchers to overcome disadvantages of traditional biologically active protein- and enzyme-based preparations, such as instability and deactivation under challenging environmental conditions, high effective dosages, and problematic delivery. Successful application of various nano- and microencapsulation technologies in medicine has generated some interest in agrotechnology. Such technologies may provide site targeted delivery, controlled release, and a prolonged life-time of agrochemicals or various macromolecules needed to improve plant resistance to pests and diseases, efficient nutrient utilization, and enhanced plant growth. In addition, use of encapsulated pesticides

may provide their less exposure to the environment and, therefore, less pollution.

In recent years, the number of publications describing the application of various encapsulation technologies in agriculture significantly increased. Various biopolymers, such as chitosan, sodium alginate, starch, and other compounds were successfully applied to encapsulate microbial inoculants and pesticides [33, 43–45]. However, as far as we know, there is no such information about encapsulation of protein- and enzyme-based agricultural preparations.

In this study, we investigated a possibility to use PHB-based microparticles as a system for protection, delivery, and controlled release of our MF3 protein. As we have mentioned earlier, microparticles of PHB and its derivatives are actively used in medicine as drug carriers. There are also some publications on the use of PHB particles of a millimeter-size range in the production of slow-release formulations for pesticides and fertilizers [46–48]. In our case, we obtained spherical 10–25-μm microparticles with aneffective loading capacity of about 50%. The used Solid/Oil/Water technology required the application of additional biopolymers, chitosan,and dextran, to stabilize MF3 within the complex. Kinetic study showed the majority of the loaded protein released in the first 24 h that could be attributed to a large amount of MF3 adsorbed on the surface layer of particles; the further 12-day release of the rest of protein was probably determined by its diffusion from the internal particle volume. These characteristics of the resulted microparticles may be determined rather by the structure and molecular mass of biopolymer [34, 49].

Since application of encapsulation technologies in relation to enzymes is often limited by the loss of enzyme activity or aggregation of resulted microparticles [42, 50], a confirmed maintenance of the plant protection properties of encapsulated MF3 represents an important outcome of the study. For both model systems used, the ability of encapsulated MF3 to suppress the development of necrotic lesions on tobacco leaves caused by infection with TMV or *A. longipes* was comparable to that of free MF3 of the same concentration. Therefore, encapsulation process does not provide negative influence on the protein activity, and MF3 successfully releases from the complex and can reach its putative receptors in plant cells.

Finally, *in vitro* assessment of the effect of UV irradiation and enzymatic proteolysis on the protecting activity of free and encapsulated MF3 showed a clear shielding effect of PHB even after an 8-h UV exposure, when free MF3 completely lost its ability to inhibit tobacco mosaic or brown rot development. The same picture was observed for proteinase K treatment: free MF3 significantly lost its activity, while encapsulated MF3 kept the most part of its protecting efficiency even in the case of a relatively high proteinase K concentration. Thus, the study demonstrated a good shielding potential of PHB as a part of MF3-based complexes against adverse biotic and abiotic factors.

4. CONCLUSIONS

Results of our study confirmed the possibility to develop environmentally friendly biopesticides based on a biopolymeric complex of MF3 and PHB and characterized by the improved resistance of its bioactive component to adverse biotic and abiotic factors. MF3 ability to induce resistance in various plants to different pathogens and its high heat resistance, facilitating the isolation of this protein, represent additional advantages for the industrial production of MF3-based biopesticides. In the further work, we will arrange some additional experiments on the microsphere production to improve the effective loading capacity and to obtain more gradual release rate for MF3 and will continue the study of characteristics of encapsulated MF3 using other model systems and whole plants.

5. REFERENCES

[1] Oerke E.C., Crop losses to pests. *J. Agr. Sci.*, 144, 1, 31-43, **2006.**

[2] Cooke L.R., Schepers H.T.A.M., Hermansen A., Bain R.A., Bradshaw N.J., Ritchie F., Shaw D.S., Evenhuis A., Kessel G.J.T., Wander J.G.N., Andersson B., Hansen J.G., Hannukkala A., Nærstad R., Nielsen B.J., Epidemiology and integrated control of potato late blight in Europe. *Potato Res.*, 54, 2, 183–222, **2011.**

[3] Maffei M.E., Arimura G.I., Mithoefer A., Natural elicitors, effectors and modulators of plant responses. *Nat. Prod. Rep*., 29, 1288–1303, **2012.**

[4] Siah A., Magnin-Robert M., Randoux B., Choma C., Rivière C., Halama P., Reignault P. Natural agents inducing plant resistance against pests and diseases, *Natural antimicrobial agents, sustainable development and biodiversity* (Mérillon J.-M., Rivière C., eds.). Springer, 121–159, **2018.**

[5] Mejia-Teniente L., Torres-Pacheco I., Gonzalez-Chavira M.M., Ocampo-Velazquez R.V., Herrera-Ruiz G., Chapa-Oliver A.M., Guevara-González R.G., Use of elicitors as an approach for sustainable agriculture. *Afr. J. Biotechnol.*, 9, 9155–9162, **2010.**

[6] Dodds P.N., Rathjen J.P., Plant immunity: towards an integrated view of plant-pathogen interactions, *Nat. Rev. Genet*., 11, 539–548, **2010.**

[7] Djavakhia V.G., Nikolaev O.N., Voinova T.M., Battchikova, N.V., Korpela T., Khomutov, R.M., DNA sequence of gene and amino acid sequence of protein from *Bacillus thuringiensis*, which induces non-specific resistance of plants to viral and fungal diseases, *J. Russ. Phytopathol. Soc*., 1, 1563–3683, **2000.**

[8] Felix G., Boller T., Molecular sensing of bacteria in plants. The highly conserved RNA-binding motif RNP-1 of bacterial cold shock proteins is recognized as an elicitor signal in tobacco, *J. Biol. Chem*., 278, 6201–6208, **2003.**

[9] Zipfel C., Robatzek S., Navarro L., Oakeley E.J., Jones J.D.G., Felix G., Boller T., Bacterial disease resistance in Arabidopsis through flagellin perception, *Nature*, 428, 764–767,**2004.**

[10] Yang M., Ji X., Studies on the induce-resistance effect of harpin on downy mildew and *Botrytis cinerea* of cucumber, *Chin. Agric. Sci. Bull.*, 25, 228–230, **2009.**

[11] Furukawa T., Inagaki H., Takai R., Hirai H., Che F.S., Two distinct EF-Tu epitopes induce immune responses in rice and Arabidopsis, *Mol. Plant Microbe Interact*., 27, 2, 113–24, **2014.**

[12] Park H.Y., Park H.C., Yoon M.Y. Screening for peptides binding on *Phytophthora capsici* extracts by phage display, *J. Microbiol. Meth.*, 78, 54–58, **2009.**

[13] Peng D.H., Qiu D.W., Ruan L.F., Zhou C.F., Sun M., Protein elicitor PemG1 from *Magnaporthe grisea* induces systemic acquired resistance (SAR) in plants, *Mol. Plant Microbe Interact*., 24, 10, 1239–1246, **2011.**

[14] Kulye M., Liu H., Zhang Y., Zeng H., Yang X., Qiu D. Hrip1, a novel protein elicitor from necrotrophic fungus, *Alternaria tenuissima*, elicits cell death, expression of defence-related genes and systemic acquired resistance in tobacco,*Plant Cell Environ.*, 35, 12, 2104–2120, **2012.**

[15] Wang B., Yang X., Zeng H., Liu H., Zhou T., Tan B., Yuan J., Guo L., Qiu D., The purification and characterization of a novel hypersensitive-like response-inducing elicitor from *Verticillium dahliae* that induces resistance responses in tobacco, *Appl. Microbiol. Biotechnol*., 93, 1, 191–201, **2012.**

[16] Hael-Conrad V., Abou-Mansour E., Díaz-Ricci J.C., Métraux J.P., Serrano M., The novel elicitor AsES triggers a defense response against *Botrytis cinerea* in *Arabidopsis thaliana, Plant Sci.*, 241, 120–127, **2015.**

[17] Wang H., Yang X., Guo L., Zeng H., Qiu D., PeBL1, a novel protein elicitor from *Brevibacillus laterosporus* strain A60, activates defense responses and systemic resistance in *Nicotiana benthamiana*,*Appl. Environ. Microbiol*., 81, 8, 2706–2716, **2015.**

[18] Nováková M., Kim P.D., Šašek V., Burketová L., Jindřichová B., Šantrůček J., Valentová O., Separation and identification of candidate protein elicitors from the cultivation medium of *Leptosphaeria maculans* inducing resistance in *Brassica napus*, *Biotechnol. Prog*., 32, 4, 918–928,**2016.**

[19] Shcherbakova L.A., Odintsova T.I., Stakheev A.A., Fravel D.R., Zavriev S.K., Identification of a novel small cysteine-rich protein in the fraction from the biocontrol *Fusarium oxysporum*strain CS-20 that mitigates fusarium wilt symptoms and triggers defense responses in tomato, *Front. Plant Sci*., 6, 1–15,**2016.**

[20] Qiu D., Dong Y., Zhang Y., Li S., Shi F., Plant immunity inducer development and application, *Mol. Plant Microbe Interact.*, 30, 5, 355–360, **2017.**

[21] Fontanilla M., Montes M., De Prado R., Effects of the foliar-applied protein "Harpin(Ea)" (Messenger) on tomatoes infected with *Phytophthora infestans*, *Commun. Agric. Appl. Biol. Sci*., 70, 3, 41–45, **2005.**

[22] Tubajika K.M., Civerolo E.L., Puterka G.J., Hashim J.M., Luvisi D.A., The effects of kaolin, harpin, and imidacloprid on development of Pierce's disease in grape, *Crop Prot*., 26, 92–99, **2007.**

[23] Wang J., Bi Y., Wang Y., Deng J., Zhang H., Zhang Z., Multiple preharvest treatments with harpin reduce postharvest disease and maintain quality in muskmelon fruit (cv. Huanghemi), *Phytoparasitica*, 42, 155–163, **2014.**

[24] Díaz-Pérez J.C.,*Effect of harpin protein ('Messenger') on bell pepper plant growth, plant disease, and fruit yield. 2007 Extension Research Report*, **2007** URL: https://athenaeum.libs.uga.edu/bitstream/handle/10724/33684/CM1-Diaz.pdf?sequence=1&isAllowed=y Accessed Apr 19, 2018.

[25] Maketon C., Tongjib Y., Patipong T., Meechareon N., Rungratanaubon T., Maketon M., Greenhouse evaluations of harpin protein and microbial fungicides in controlling *Curvularia lunata*, *Fusarium moniliforme*, and *Phythopthorapalmivora*, major causes of orchid diseases in Thailand, *Life Sci. J.*, 12, 4, 125–132, **2015.**

[26] Zhang W., Yang X., Qiu D., Guo L., Zenq H., Mao J., Gao Q., PeaT1-induced systemic acquired resistance in tobacco follows salicylic acid-dependent pathway, *Mol. Biol. Prep.*, 38, 4, 2549–2556, **2011.**

[27] Shumilina D., Kramer R., Klocke E., Dzhavakhiya V., MF3 (peptidyl-prolyl cis-trans isomerase of FKBP type from *Pseudomonas fluorescens*) – an elicitor of non-specific plant resistance against pathogens, *Phytopathol. Pol*., 41, 39–49, **2006.**

[28] Shumilina D.V., Dzhavakhiya V.G., Study of the ability of MF3 protein (FKBP-type peptidyl prolyl cis-trans isomerase) from *Pseudomonas fluorescens* to improve resistance of tobacco plants to viral and fungal pathogens, *Agro XXI*, 1, 7–9, **2007.**

[29] Dzhavakhiya V.G., Voinova T.M., Shumilina D.V., Search for the active center of peptidyl prolyl cis/trans isomerase from *Pseudomonas fluorescens* responsible for the induction of tobacco (*Nicotiana tabacum* L.) plant resistance to tobacco mosaic virus, *Agr. Biol.*, 51, 3, 392–400, **2016.**

[30] Walters D.R., Fountain J.M., Practical application of induced resistance to plant diseases: and appraisal of effectiveness under field conditions, *J. Agr. Sci.*, 147, 523–535, **2009.**

[31] Zhao H., Lin Z.Y., Yildirimer L., Dhinakar A., Zhao H., Wu J., Polymer-based nanoparticles for protein delivery: design, strategies, and applications, *J. Mater. Chem. B*, 4, 4060–4071, **2016.**

[32] Roy A., Singh S.K., Bajpai J., Bajpai A.K., Controlled pesticide release from biodegradable polymers, *Central Eur. J. Chem.*, 12, 4, 453–469, **2014.**

[33] Vemmer M., Patel A.V., Review on encapsulation methods suitable for microbial biological control agents, *Biol. Control*, 67, 3, 380–389, **2016.**

[34] Bonartsev A.P., Bonartseva G.A., Shaitan K.V., Kirpichnikov M.P., Poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate)-based biopolymer systems, *Biochem. (Mosc.) Suppl. Ser. B*, 5, 1, 10–21, **2011.**

[35] Dzhavakhiya V., Filipov A., Skryabin K., Voinova T., Kouznetsova M., Shulga O., Shumilina D., Kromina K., Pridannikov M., Battchikova N, Korpela T. Proteins inducing multiple resistance of plants to phytopathogens and pests, Int. Patent Application WO2005/061533, **2005.**

[36] Popletaeva S.B., Statsyuk N.V., Voinova T.M., Arslanova L.R., Zernov A.L., Bonartsev A.P., Bonartseva G.A., Dzhavakhiya V.G., Evaluation of eliciting activity of peptidil-prolyl cys/trans isomerase from *Pseudonomas fluorescens* encapsulated in sodium alginate regarding plant resistance to viral and fungal pathogens, *AIMS Microbiol*., 4, 1, 192–208, **2018**.

[37] Bonartsev A., Yakovlev S., Boskhomdzhiev A., Zharkova I., Bagrov D., Myshkina V., Mahina T., Kharitonova E., Samsonova O., Zernov A., Zhuikov V., Efremov Y., Voinova V., Bonartseva G., Shaitan K., The terpolymer produced by *Azotobacter chroococcum* 7B: effect of surface properties on cell attachment, *Plos One*, 8, 2, e57200, **2013.**

[38] Castellanos I.J., Griebenow K., Improved α-chymotrypsin stability upon encapsulation in PLGA microspheres by solvent replacement, *Pharm. Res*., 20, 11, 1873–1880, **2003.**

[39] Van S.N., Minh H.D., Anh D.N., Study on chitosan nanoparticles on biophysical characteristics and growth of Robusta coffee in green house, *Biocatal. Agr. Biotechnol.*, 2, 4, 289–294, **2013.**

[40] Shagdarova B.T., Ilyina A.V., Lopatin S.A., Kartashov M.I., Arslanova L.R., Dzhavakhiya V.G., Varlamov V.P. Study of the protective activity of chitosan hydrolyzate against septoria leaf blotch of wheat and brown spot of tobacco, *Appl. Biochem. Microbiol*., 54, 1, 71–75, **2018.**

[41] Xing K., Zhu X., Peng X., Qin S., Chitosan antimicrobial and eliciting properties for pest control in agriculture: a review. *Agron. Sustain. Dev*., 35, 2, 569–588, **2015.**

[42] Kiseleva R., Vertegel A.,Polyester carriers for enzyme delivery. *Handbook of polyester drug delivery systems* (Ravikumar M.N.V., ed.), Singapore: Pan Stanford, 675–709, **2016.**

[43] Schoebitz M., López M.D., Roldán A., Bioencapsulation of microbial inoculants for better soil-plant fertilization, A review. *Agron. Sustain. Dev*., 33, 751–765, **2013.**

[44] Kashyap P.L., Xiang X., Heiden P., Chitosan nanoparticle based delivery systems for sustainable agriculture, *Int. J. Biol. Macromol.*, 77, 36–51, **2015.**

[45] Vinceković M., Jalšenjak N., Topolovec-Pintarić S., Đermić E., Bujan M., Jurić S., Encapsulation of biological and chemical agents for plant nutrition and protection: chitosan/alginate microcapsules loaded with copper cations and *Trichoderma viride*, *J. Agric. Food Chem.*, 64, 43, 8073–8083, **2016.**

[46] Boyandin A.N., Zhila N.O., Kiselev E.G., Volova T.G., Constructing slow-release formulations of metribuzin based on degradable poly(3-hydroxybutyrate), *J. Agric. Food Chem.*, 64, 28, 5625–5632, **2016.**

[47] Volova T.G., Prudnikova S.V., Boyandin A.N., Biodegradable poly-3-hydroxybutyrate as a fertiliser carrier, *J. Sci. Food Agric.*, 96, 12, 4183–93, **2016.**

[48] Volova T.G., Prudnikova S.V., Zhila N.O., Vinogradova O.N., Shumilova A.A., Nikolaeva E.D., Kiselev E.G., Shishatskaya E.I., Efficacy of tebuconazole embedded in biodegradable poly-3-hydroxybutyrate to inhibit the development of *Fusarium moniliforme* in soil microecosystems, *Pest Manag. Sci.*, 73, 5, 925–935, **2017.**

[49] Gaspar M.M., Blanco D., Cruz M.E.M., Alonso M.J., Formulation of L-asparaginase-loaded poly(lactide-co-glycolide) nanoparticles: influence of polymer properties on enzyme loading, activity and *in vitro* release, *J. Control. Release*, 52, 53–62, **1998.**

[50] Castellanos I.J., Cruz G., Crespo R., Griebenow K., Encapsulation-induced aggregation and loss in activity of gamma-chymotrypsin and their prevention, *J. Control. Release*, 81, 3, 307–319, **2002.**

6. ACKNOWLEDGEMENTS

The study was carried out within the framework of the state assignment of the Russian Federal Agency for Scientific Organizations (theme no. 0598-2016-0001). Part of the work, namely, production of microspheres, was carried out within the framework of the state assignment of the Russian Federal Agency for Scientific Organizations (theme no. 01201351358) using the equipment of the Collective Use Center of the Federal Research Center of Biotechnology, Russian Academy of Sciences.

Strains of plant pathogenic microorganisms used in this study were provided by the Collective Use Center "State Collection of Plant Pathogenic Microorganisms, Indicator Plants and Differential Cultivars" of the All-Russian Research Institute of Phytopathology.