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GENE CONSTRUCT-BASED SERINE PROTEASE OF *Bacillus pumilus* AS A FEED ADDITIVE FOR POULTRY FARMING

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Abstract

Bacterial enzymes, in particular proteinases, as dietary additives in poultry farming, improve digestibility of feed nutrients and, as a consequence, make animal diets cheaper. This explains why bioadditives are being actively developed worldwide. Proteinases break down proteins and reduce the negative effect of digestive inhibitors thus allow the costs for purchasing synthetic amino acids to be lower. Bacteria and microscopic fungi, including those with gene constructs developed to increase yield and improve properties of the expressed enzymes, may be producers. Bacterial serine proteinases have a high thermostability and are resistant to inhibitors of animal origin. In this paper, we report for the first time about the production of highly purified secreted subtilisin-like serine proteinase from *Bacillus pumilus* upon expression of the recombinant vector in *B. subtilis* strains and evaluate the main physicochemical and biological characteristics of the synthesized product. The goal of our study is to obtain, by using the expression system, the highly purified subtilisin-like serine proteinase from *B. pumilus* as a promising feed additive for the poultry industry. The substrate specificity of the produced serine proteinase, i.e. the depth of hydrolysis, corresponds to the specificity of subtilisins, the enzyme cleaves the bonds formed by the carboxyl groups of the hydrophobic amino acids leucine, phenylalanine and tyrosine, as well as a number of hydrophilic amino acids. An investigation of the effect of temperature and pH on serine proteinase activity showed that in the presence of calcium ions at a final concentration of 5 mM, the temperature optimum of the enzyme reached 50 °C. The enzyme remaines stable in the pH range from 7 to 10. The proteinase activity was studied at various pH values to simulate the conditions of the gastrointestinal tract of chickens. In a weakly acidic medium (pH 5.5, goiter) proteinase completely retains its activity (100 %), at pH 2.9 (stomach) the enzyme activity decreases by 40 %, and upon transition again to alkaline conditions (pH 6.5-8.0, small intestine and large intestine), the enzyme restores activity up to the values exceeding control by 13 %. Thus, the enzyme can remain active throughout the whole digestive tract of broiler chicks. The proteinase activity was not inhibited by natural inhibitors, such as a trypsin inhibitor, which would also allow the enzyme to function in the gastrointestinal tract of chickens. In experiments on the effect of chicken bile from 0.01 % to 0.05 % for 1 hour at 40 °C on the microbial proteinase, the enzyme completely preserved its activity. With an increase in the concentration of chicken bile to 1 %, the enzyme activity decreased by 10 %. To study the toxicity of proteinase, 1-day-old Cobb 500 broiler chickens were observed for 10 days. The dietary proteinase at 100 EU/kg concentration showed no toxicity, and all the indices of the poultry remained normal. We found that in the early period, during 0-10 days of growth when the chickens are fed with Start ration, a dosage of 5 EU/kg of proteinase is effective. In the late stages of poultry growth (21-42 days), the use the Finisher mixed feed supplemented with bacterial proteinase at a dose of 15 EU/kg is optimal. In both cases, the dietary proteinase increases poultry weight gain by 13.9 % and 7.9 %, and also improves feed conversion by 14 % and

8.5 %, respectively. Thus, the amount of the introduced enzyme must be adjusted depending on the age of birds and the feed composition. The main indicators of Cobb 500 broiler chickens' growth when using recombinant proteinase allow us to conclude that this proteinase is promising as a feed additive.

Keywords: *Bacillus pumilus*, recombinant subtilisin-like serine proteinase, substrate specificity, stability, activity, effects of pH and temperature, fodder additive, broiler chickens, Cobb 500.

Economic benefits from more complete assimilation of cereal feeds by increasing the digestibility of nutrients remain a pressing issue in commercial poultry farming [1-5]. Enzymes, including bacillary proteinases, can help to solve the problem. Proteinases increase the digestibility of protein components, necessary for growing broilers, and also destroy the bonds between proteins, starch or fiber, which positively affects starch digestibility by increasing its bioavailability [6-10]. The use of microbial proteases also improves the digestibility of feeds with a high content of non-starch polysaccharides [11-13]. Dietary multienzyme complexes (protease/β-glucanase/pectinase) resulted in an increase in live weight of laying hens, in egg weight, in a darker yolk, and also had a positive effect on the digestive organs [14]. In addition, exogenous proteinases, due to their effect on anti-nutritional components, for example, by destruction of the inhibitors of trypsin and lectins in soybean meal, increase the digestibility of feed nutrients [15, 16]. Exogenous proteases act as a prophylactic agent, reducing the amount of undigested protein, which is a factor in colonization of the intestine by pathogenic microorganisms, leading to the development of coccidiosis and necrotic enteritis in chickens [17, 18]. It is known that undigested proteins are factors leading to dysbacteriosis which causes necrotic enteritis [19, 20]. The protease additives improved the productivity of broilers infected by *Eimeria* spp., the causative agent of necrotic enteritis [21]. The complex preparations of living bacteria or spores in combination with exogenous proteases have a growth-promoting and protective effect on chickens [22, 23].

A search for new producers and design of effective recombinant microbial enzymes used as feed additives is an important biotechnological task [24-26]. Effective expression systems are developed to obtain feed additives based on microbial proteinases in the required quantity [27]. The inexpensive components of the media for bacilli culture, as well as the safe status of these microorganisms, determine the prospects for their use in poultry farming.

This paper is our first report on synthesis of highly purified secreted subtilisin-like serine proteinase of *Bacillus pumilus* upon expression of recombinant vectors in *B. subtilis* strains, with characterization of the main physicochemical and biological parameters of the recombinant product, which determine its promising use as a feed additive.

The goal of the study is to produce, using the expression system, highly purified subtilisin-like serine proteinase of *Bacillus pumilus* as a feed additive for poultry.

Techniques. Natural isolate *B. pumilus* 7P, its streptomycin-resistant mutant *B. pumilus* 7P/3-19, and plasmids pCS9 with gene for *B. pumilus* subtilisin-like protease were provided by S.V. Kostrov (Institute of Molecular Genetics RAS, Moscow), pGP382 was brought by courtesy of Dr. Prof. T. Mascher (Ludwig-Maximilians-Universität München, Germany). A protease-deficient strain *B. subtilis* BG 2036 (by the courtesy of Prof. E. Ferrarri, Genencor Int., Inc., USA) was a recipient. Recombinant vectors pTN 3036 (pLIKE-rep + *aprBp*), pTN 3050 (pLIKE-rep + SP_{Pac} + *aprBp*), pTN 3093 (pLIKE-rep + SP_{Yngk} + *aprBp*) and pTN 3801 (pGP382 + *aprBp*) were used to transform the protease-deficient strain *B. subtilis* BG 2036 to obtaine strains *B. subtilis* MRB044, *B. subtilis* MRB045, *B. subtilis* MRB046 and *B. subtilis* MRB072, respectively. The strains and plasmids used are stored in the museum of the Laboratory of Microbial Bio-

technology of the Kazan Federal University.

The growth medium composition was as follows (g/l): bacteriological peptone (Sigma, USA) — 20, $CaCl_2 \cdot 2H_2O$ — 0.6, $MgSO_4 \cdot 7H_2O$ — 0.5, NaCl — 3, $MnSO_4$ — 0.1, Na_2HPO_4 — 0.2, NH_4Cl — 0.2. The strains *B. subtilis* containing recombinant constructs were cultured with erythromycin and lincomycin (10 and 25 μg/μl, respectively), the recombinant strain *B. subtilis* pCS9 with 20 μg/μl erythromycin. The subtilisin-like proteinase production was evaluated by the ratio of proteolytic activity to the biomass and expressed in conditional units.

The proteinase activity was determined by the hydrolysis of azocasein (Sigma, USA) as described [28, 29]. The enzyme activity unit (EU) was the amount of enzyme hydrolyzing 1 μg of substrate per 1 min under the experimental conditions. The specificity of the proteinase was estimated by the effect on the β-chain of oxidized sheep insulin [30]. Measurements were conducted on an xMark spectrophotometer (Bio-Rad, USA).

A bioreactor Biotron LiFlus SP30L (Biotron, Inc., Korea) was used for proteinase production. Fifteen liters of the medium was sterilized in the reactor for 30 min at 121 °C; the pH of the medium was adjusted to pH 8.5 automatically and maintained by the addition of 2 N NaOH through the peristaltic system of the bioreactor. Three hundred milliliters of a 16-hour inoculum (2 % of the medium, v/v, OD_{600} 3.0), the antibiotic erythromycin (to a final concentration of 10 μg/μl) and a defoaming agent Sofeksil 1250 (Sofeks, Moscow) were introduced in the fermenter. Bacteria was cultured for 24 hours at 37 °C with constant aeration (the flow rate of 10 l/min, O_2 content is not lower than 20 %) and stirring (150-900 rpm). After 24 h growth, when the enzyme activity reached the maximum (4.4 EU/ml, OD_{600} 6.0) the cells were removed by centrifugation (5000 rpm, 15 min, Beckman Avanti JXN-26, Beckman Coulter, Inc., USA). Proteinase was purified on a column with carboxymethylcellulose (CMC) (Sigma, USA). The supernatant after the centrifugation was diluted 10 times with distilled water (pH was adjusted to 6.3), then mixed with CMC and equilibrated with 0.02 M Na-acetate buffer (pH 6.3). The mixture was kept for 90 min with constant stirring for the sorption of the enzyme. Then CMC aggregate was precipitated, the supernatant fluid was removed and the sorbent was used to fill the column. The column was rinsed with the same buffer, the protein was eluted with 0.2 M Na-acetate buffer (pH 6.3), and the enzyme activity was measured in the resulting fractions.

The molecular weight of the produced proteinase was determined by SDS-electrophoresis [31].

The physicochemical properties of the proteinase were evaluated by the temperature optimum in the presence and in the absence of calcium ions (5 mM $CaCl_2$). To assay thermostability, enzyme solutions in 0.05 M Tris-HCl buffer (pH 7.2) were incubated at the temperatures from 0 to 70 °C for 30 min, then the activity was determined at 37 °C as described above. To assess pH optimum, the enzyme activity in 0.05 M Tris-HCl buffer was determined. When studying pH stability, protein solutions were incubated in 0.05 M Tris-HCl buffer for 1 h at 25 °C, then azocasein was added and the activity was determined at 37 °C as described above.

The effect of inhibitors on a proteinase was studied using phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), o-phenanthroline (a specific inhibitor of metalloproteinases) and ovomucoid (trypsin inhibitor). The protein solution was incubated with an inhibitor for 1 h at 37 °C in Tris-HCl buffer (pH 7.2) and the proteolytic activity was determined as described above.

To imitate the conditions of the gastrointestinal tract (GIT) of chickens,

Britton-Robinson universal buffer was used [32]. A series of four aliquots of universal buffer (0.04 M) was prepared with different pH values (2.9; 5.5; 6.0; 6.3 and 8.0). The enzyme was transferred from one solution to another by the method of successive dilutions and kept in each for an appropriate time; total enzyme dilution 1:200. The sequence of aliquots corresponded to the sequence of the digestive tract sections of chickens: pH 5.5 (50 min), ingluvies simulation; pH 2.9 (90 min), stomach simulation; pH 6.5 (30 min), small intestine simulation, pH 8 (70 min), large intestine simulation. The buffer temperature throughout the experiment was 40 °C.

Bile, obtained from 10-day-old broiler chickens, was diluted with 0.02 M Na-acetate buffer (pH 6.3). The samples containing bile in the enzyme solution from 0.01 % to 5 % were kept at 40 °C for 60 min. The samples were taken every 15 min to measure the activity. An enzyme solution in 0.02 M Na-acetate buffer (pH 6.3) without bile was control. The control solution of the enzyme was kept at 40 °C for 1 h and the proteinase activity was determined.

The properties of proteinase as a feed additive were studied under the conditions of a peasant farm enterprise (Srednee Azyakovo village, Medvedevskii District, Mari-El Republic). For the experiment, 225 1-day-old Cobb 500 chickens with an average live weight of 0.049±0.003 kg were selected, out of which a control group (75 birds) was formed, which received standard mixed feed ration, and two experimental groups (75 birds each), where recombinant proteinase was added to the mixed feed at a dose of 5 EU/kg (group I) or 15 EU/kg (group II). The experiment continued for 42 days. The chickens aged 0-10 days received the Start mixed feed, 11-20 days — the Growth, 21-42 days — the Finisher feeds in accordance with growing technologies (Algoritm investitsii LLC, Yoshkar-Ola, Mari El Republic). The enzyme solution was added to dry feed by spraying with constant stirring. The chickens were kept in ventilated cellular batteries at 35-36 °C. Weight gain in chickens was evaluated daily, from the initial to the final day of the experiment. The amount of the consumed feed was counted per chicken. The feed conversion ratio was calculated as the ratio of the amount of consumed feed to the increase in body weight.

The toxicity of the proteinase preparation was studied on 1-day-old Cobb 500 chickens weighing 0.047±0.001 kg, out of which a control group (15 birds) was formed, which received only mixed feed, and an experimental group (15 birds), where proteinase was added (100 EU/kg feed) for 10 days. During the experiment, the chickens were weighted, and their behavior was controlled and excrements were surveyed. After 10 days, 3 chickens were randomly selected from each group and euthanized by inhalation anesthesia with chloroform to examine the internal organs.

The statistical processing of the results included the calculation of the mean value (M) and the standard error of the mean (±SEM). The significance of differences was assessed by Student's t-test. The differences were considered statistically significant at $p < 0.05$.

Results. We compared the expression of subtilisin-like extracellular serine proteinase of *B. pumilus* in natural and recombinant strains. The wild-type strain *B. pumilus* 7P is a natural isolate with an increased production of extracellular ribonuclease and other enzymes, including the proteinase, *B. pumilus* 7P/3-19 is its streptomycin-resistant mutant. A recombinant strain *B. subtilis* pCS9 was obtained, which carries the multicopy plasmid pCS9 containing the proteinase gene *B. pumilus* (*aprBp*) with its own signal peptide under the control of its own promotor. To clone *aprBp*, an optimized LIKE expression system based on the promotor *liaI B. subtilis* was also used, which is regulated by a two-component antibiotic-induced system LiaRS [33, 34]. As part of the pLIKE-rep vector, the

gene *aprBp* was introduced into *B. subtilis* MRB044 with its own signal peptide (pTN 3036, pLIKE-rep + *aprBp*), into *B. subtilis* MRB045 with the nucleotide sequence of the signal peptide of penicillin-amidase (penicillin amidohydrolase, EC 3.5.1.11) gene of *B. megaterium* (pTN 3050, pLIKE-rep + SP_{Pac} + *aprBp*), and into *B. subtilis* MRB046 with the sequence of the recombinant signal peptide of glycoside hydrolase (EC 3.2.1.-) gene of *B. megaterium* (pTN 3093, pLIKE-rep + SP_{Yngk} + *aprBp*). pGP382 expression vector with a strong constitutive promoter (P_{DegQ}) [35] was also used to clone *aprBp* gene [35]. The *degQ* gene encodes the protein (46 amino acid residues) involved in the phosphorylation of the two-component system DegS/DegU that controls the synthesis of proteinases [36]. *B. subtilis* MRB072 contained plasmid pGP382 with the *aprBp* gene and the Strep tag in the composition of a protein affinity purification vector (pTN 3801, pGP382 + *aprBp*). The comparison of expression showed *B. subtilis* pCS9 (Table 1) to be the most effective proteinase producer, which was used to continue the experiments.

1. Proteolytic activity in the culture medium of natural isolates *Bacillus* and recombinant strains with *B. pumilus* subtilisin-like serine proteinase gene *aprBp* in various constructions ($n = 10$)

Strain	Activity, EU	Productivity, units
B. pumilus 7P	1.50±0.02	0.50±0.01*
B. pumilus 7P/3-19	2.90±0.01	0.93±0.02*
B. subtilis pCS9	3.50±0.01	1.14±0.02*
B. subtilis MRB044	0.25±0.05	0.10±0.01
B. subtilis MRB045	0.30±0.02	0.12±0.02
B. subtilis MRB046	0.42±0.03	0.17±0.02*
B. subtilis MRB072	0.50±0.01	0.20±0.04

* Differences with control are statistically significant at $p < 0.05$.

2. Purification of recombinant subtilisin-like proteinase expressed in *Bacillus subtilis* pCS9 with *B. pumilus* gene *aprBp* in the plasmid pCS9 ($n = 5$)

Stage of purification	Volume, ml	Protein mg/ml	Activity			Purity	Yield, %
			EU/ml	total, EU	specific, EU/mg		
Culture medium	12800	870±20*	4.4±0.07*	56320[a]	0.005	1.0	100
Ion-exchange chromatography on carboxymethylcellulose	470	383±10*	33.8±0.1*	15886[a]	0.088	17.6	28.2

N o t e. [a] — mean values of activity.

* Differences with control are statistically significant at $p < 0.05$.

After culture of *B. subtilis* pCS9 in the bioreactor and purification of the proteinase, a preparation with total activity of 15886 EU was obtained from joined electrophoretic fractions with high activity (Table 2). SDS electrophoresis confirmed the presence of 28 kDa protein. Thus, using CMC chromatography, a highly purified recombinant subtilisin-like proteinase preparation was obtained in 0.2 M Na-acetate buffer (pH 6.3). Since the buffer components are non-toxic to chickens, their presence in the enzyme solution was not an obstacle for the use in experiments with poultry.

The temperature optimum of the recombinant enzyme was 37 °C (Fig. 1, A). For the practical use, it is important that in the presence of calcium ions at a final concentration of 5 mM, the temperature optimum of the enzyme increased to 50 °C. In our experiment, the enzyme activity increased on average by 40 % at 50 °C and by 60 % at 55 °C (see Fig. 1, B). The proteinase remained stable in the temperature range from 0 to 40 °C. The optimum acidity was pH 9.5. The proteinase retained stability in the range of pH 7-10. At pH 3 and pH 11, the drop in activity did not exceed 40 % (see Fig. 1, B). The data on thermo- and pH stability of the protein testify to the possibility of its use as a feed additive. When distilling an aliquot of the enzyme with a solution of a specific inhibitor of serine proteinas-

es PMSF (1:1000), the enzymatic activity was completely suppressed, and it did not change in the presence of metalloproteinase inhibitors EDTA and o-phenanthroline (1:100). These data indicate that the enzyme belongs to the class of serine proteinases. The proteinase activity was not inhibited by the trypsin inhibitor, thence, it was suggested that the recombinant enzyme will be able to function in the digestive tract of chickens.

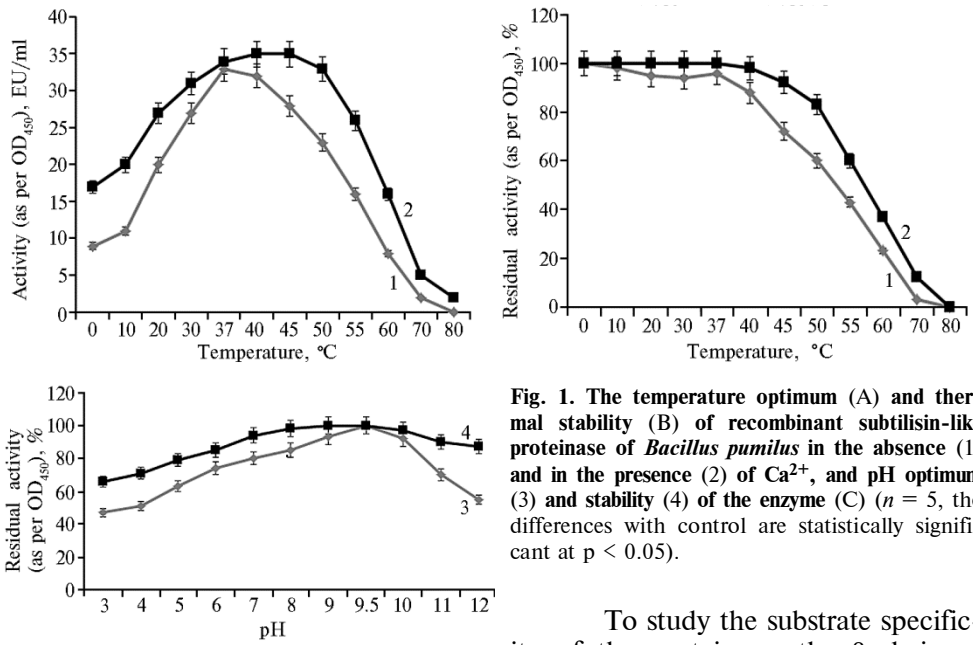

Fig. 1. The temperature optimum (A) **and thermal stability** (B) **of recombinant subtilisin-like proteinase of *Bacillus pumilus* in the absence** (1) **and in the presence** (2) **of Ca^{2+}, and pH optimum** (3) **and stability** (4) **of the enzyme** (C) (n = 5, the differences with control are statistically significant at $p < 0.05$).

To study the substrate specificity of the proteinase, the β-chain of the oxidized insulin was used. The hydrolysis of the of_the β-chain led to numerous peptide fragments detected by thin-layer chromatography (the data not given), indicating a broad substrate specificity well-known for subtilisin-like enzymes, e.g. proteinase K, esperase of *B. lentus* and subtilisin BPN′ of *B. amyloliquefaciens* [37]. The enzyme hydrolyzes the bonds formed by carboxyl groups of hydrophobic amino acids (Phe1-Val2, Leu11-Val12, Leu15-Tyr16, Phe25-Tyr26, etc.) and also hydrophilic amino acids (Asn3-Gln4, Gln4-His5, Cys7-Gly8, Ser9-His10, Tyr16-Leu17, etc.). Consequently, the obtained proteinase of *B. pumilus* has wide substrate specificity and the ability to deeply hydrolyze protein substrates, which also determines the perspective nature of the enzyme as a bioadditive splitting protein components of feeds.

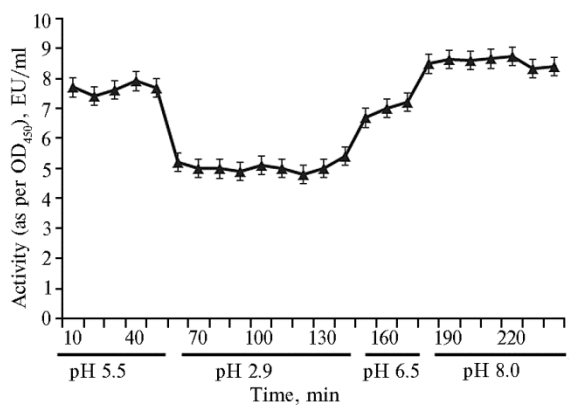

Fig. 2. The activity of the recombinant subtilisin-like proteinase of *Bacillus pumilus* at different pH values, simulating conditions in the gastrointestinal tract of chickens (n = 5, the differences with the control are statistically significant at $p < 0.05$).

To work effectively in the digestive tract of poultry, proteinase should remain active at elevated temperatures (40 °C) and aggressive pH values, ranging from an acidic to an alkaline one. The experiment with simulating the gastrointestinal condi-

tions of chickens (pH, time and temperature) showed that proteinase successfully functions in such conditions (Fig. 2). In a weakly acidic medium (pH 5.5), the enzyme remained activity within the control one. In a strongly acidic medium (pH 2.9, stomach simulation), the enzyme activity decreased by 40 %, and in alkaline conditions (pH 6.5-8.0, small and large intestine simulation) it increased by 10-13 % compared to the control. These data showed that proteinase can remain active throughout the entire digestive tract of poultry.

The study of the activity and stability of the enzyme under the action of bile for 1 h at 40 °C showed that at its concentration from 0.01 to 0.05 % the enzyme activity remained within the control value. When increasing the concentration up to 1 %, the enzyme activity decreased by 10 %, and at a concentration of 5 %, the residual activity of the enzyme was 60 % (Fig. 3). Therefore, the resulting bacterial enzyme is able to maintain catalytic activity when exposed to bile under the conditions of the gastrointestinal tract of chickens.

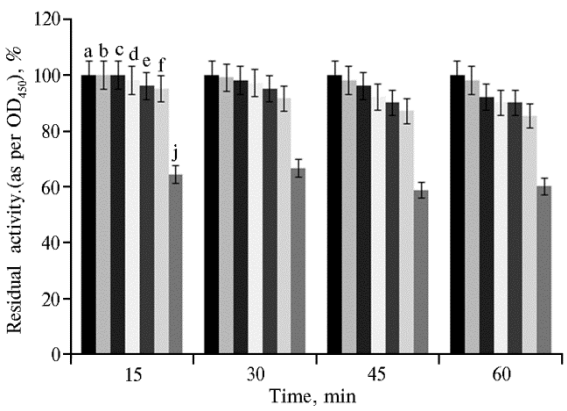

Fig. 3. The activity of recombinant subtilisin-like proteinase of *Bacillus pumilus* at different concentrations of bile: a — 0.01 %, b — 0.05%, c — 0.10%, d — 0.25%, e — 0.50%, f — 1%, g — 5% (n = 5, the differences with the control are statistically significant at $p < 0.05$).

When assessing the toxicity of the preparation, the chickens were kept in cages. The chickens were preliminarily examined by a veterinary specialist to identify the sick and weakened ones (they were excluded from the experiment).

3. The main zootechnical indicators in Cobb 500 broiler chickens upon the addition of recombinant proteinase of *Bacillus pumilus* in the feed (M±SEM, physiological experiment, a peasant farm enterprise, Mari El Republic)

Indicator	Control (n = 25)	Group I, 5 EU/kg feed (n = 25)	Group II, 15 EU/kg feed (n = 25)
Live weight gain, kg:			
0 day	0.049±0.003	0.049±0.003	0.049±0.003
1-10 days	0.201±0.007	0.229±0.008	0.214±0.005
11-20 days	0.364±0.014	0.402±0.014	0.391±0.010
21-42 days	1.551±0.032	1.668±0.038	1.674±0.039
Total	2.165±0.044	2.348±0.044	2.328±0.037
Consumption of feed per chicken, kg:			
Start (0-10 days)	0.343	0.336	0.347
Growth (11-20 days)	0.729	0.705	0.719
Finisher (21-42 days)	3.112	3.127	3.071
Total	4.184	4.168	4.137
Conversion of the feed:			
Start (0-10 days)	1.71	1.47	1.62
Growth (11-20 days)	2.00	1.75	1.84
Finisher (21-42 days)	2.01	1.88	1.84
Total	1.98	1.81	1.82
Poultry survival	100 %	100 %	100 %

N o t e. In group I and group II, the subtilisin-like proteinase of *Bacillus pumilus* was added to the main (control) ration (see *Techniques* section). The differences with the control are statistically significant at $p < 0.05$.

The Start feed was used in the form of grits. The proteinase concentration was 12.5 ml/kg feed per 15 birds. For 10 days (the period of observation), all chickens remained healthy, active, ate the feed well, physiological abnormalities and the changes in behavioral reactions were not observed. The live weight

of the chickens in the experiment remained within the control. The excrements of broiler chickens were normal. After the postmortem examination, damage and pathological changes were not revealed in the internal organs. These results confirmed that the bacterial proteinase preparation was safe and not toxic for the poultry.

When determining the nutritional value of the feeds (Growth, Start, Finisher) in adding bacterial proteinase, the calcium content (about 1 %) was sufficient to stabilize the activity of the introduced enzyme. For 42 days (the period of observation), all chickens remained healthy, active, ate the feed well, their behavioral reactions did not change. The preservation of livestock was 100 % in the control and experimental groups. By the end of fattening, the live weight of the poultry, which was given proteinase as an additive, was higher compared to control, i.e. in group I (5 EU/kg) by 8.7 % ($p < 0.05$), and in group II (15 EU/kg) by 7.7 % ($p < 0.05$) (Table 3). During 0-10 days (Start feed), the increase in the live weight of chickens in group I and group II was higher than in the control by 13.9 % ($p < 0.05$) and 6.5 % ($p < 0.05$), respectively (see Table 3). The feed conversion improved in both groups (by 14.0 and 5.3 %, respectively, $p < 0.05$). From day 11 to day 20 (Growth feed), the weight gain in group I was 10.4 % higher ($p < 0.05$), in group II 7.4 % higher ($p < 0.05$) compared to the control. The feed conversion improved (by 12.5 and 8.0 %, respectively, $p < 0.05$). When using the Finisher feed (21-42 days), there was a 7.5 % gain in group I ($p < 0.05$), and a 7.9 % gain in group II ($p < 0.05$). During this period, the feed conversion in the experimental groups improved by 6.5 and 8.5%, respectively ($p < 0.05$).

Thus, subtilisin-like proteinase of *Bacillus pumilus*, expressed in *Bacillus subtilis* pCS9 with *aprBp* gene in pCS9 plasmid, shows wide substrate specificity, stability (withstands the fluctuations of pH, temperature, high concentrations of bile), high activity (is capable of remaining activity in both the upper and the lower intestines of Cobb 500 chickens) and non-toxicity to poultry. These properties are necessary in the conditions gastrointestinal tract of broilers, since the enzyme should remove the substrate that could disturb the digestion and microflora balance as the chyme moves along the whole intestine. The obtained data allows conclusion that in the early stages of growth (0-10 days) with the use of the Start feed, the effective dose of proteinase is 5 EU/kg feed (there is a tendency to improving the feed conversion). The same is noted later (21-42 days) with the addition of proteinase at a dose of 15 EU/kg to the Finisher feed. The resulting recombinant bacillary proteinase can be considered as a potential feed additive to increase the live weight gain and reduce the feed consumption when growing broiler chickens.

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