Comparative evaluation of biostimulator efficiency on corn seeds germination: keratin protein and preparation Ribav Extra

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Abstract. Recently, great interest in agricultural production has been manifested to preparations of biological origin. Production of biostimulants is in great demand, as small concentrations of these substances are able to maximize the potential of plants, increase their quality and productivity. One of the promising sources of biostimulants is keratin-containing raw materials. It is characterized by a high content of keratin protein. The peculiarities of its chemical composition and structural and mechanical properties require a concretization of the approach to the methods of processing and processing of this unconventional type of waste. Due to enzymatic hydrolysis of keratin preparation of actinomycete Str. chromogenes s. g. 0832 managed to obtain a highly effective biostimulator for plants. The content of free amino acids in it is 82.2%. Hydrolysate contains all essential amino acids; the sum of essential amino acids was 42.66 %. In the comparative evaluation of the biostimulator from keratin pen and the preparation of plant origin "Ribav Extra" the best results were obtained when processing corn seeds with hydrolysate of animal origin. The germination energy in this case was higher than the control samples by 18%, germination increased by 10% compared to the control and by 5% compared to Ribav Extra.

1. Introduction

Ecosystem degradation is forcing the introduction of highly productive, environmentally friendly biotechnologies in agricultural production. In this regard, there is increasing interest in organic farming. Demand for organic products outstrips supply. This, in turn, arouses interest in substances that, on the one hand, are safe for the environment, and on the other hand - increase the yield and quality of crops. Such substances include biostimulants. These are substances that stimulate the natural processes of assimilation of nutrients, increasing the stress resistance of plants.

Biostimulants were divided into 3 groups [1]: humic substances, seaweed extracts, amino acids, and peptides. The source of the latter can be waste of plant and animal origin. In this regard, special attention deserves keratin-containing raw materials (source of animal protein keratin). Keratin-containing raw materials in chemical composition is a protein concentrate (90-95%) with a high content of essential
amino acids [2,3]. But because of the low functionality of proteins of this raw material (insolubility in water, high mechanical strength, inaccessibility to the action of digestive enzymes, etc.), the use of this protein is limited. This is a property of keratin it is explained by the presence of a large number of transverse disulfide bonds between polypeptide chains [4,5]. When these bonds are broken, keratin loses its stability. The difficulty is to translate this protein into a soluble state, while retaining all the amino acids of the protein. In this case, the most acceptable biotechnological methods using specific enzymes that destroy the compact structure of the keratin molecule to the digestible components. The solution of the problem will allow obtaining a biostimulator for plants with a high content of amino acids.

Many studies [6,7] indicate a positive effect of protein hydrolysates on the growth and yield of crops. Cerdan M., et al. [8], Ertani A., et al. [9] note an increase in nutrient uptake in maize and tomatoes after treatment with vegetable protein hydrolysates. Mukatova M. D. et al. [10] a biostimulator was obtained from chitin of shell-containing raw materials of river crayfish of the Volga-Caspian basin. Treatment of watermelon seeds with the obtained biostimulator increased germination and seed germination energy. But at the same time, other authors [11,12] noted that hydrolysates of animal origin can cause phytotoxicity and inhibition of plant growth. They attribute this to a higher content of free amino acids (especially proline and glycine).

Due to the contradictory data obtained by different authors, the purpose of our experiment was to determine the effect of the biostimulator obtained by enzymatic hydrolysis of animal protein keratin in comparison with the biostimulator of plant origin.

2. Materials and methods
As a biostimulant of plant origin we used the drug "Ribav Extra". It is a 60% alcohol extract of metabolic products of mycorrhizal fungi isolated from the roots of ginseng (produced by LLC Biotech center "Ribav", Russia). Biostimulant of animal origin serves as a hydrolysate of keratin protein pen. The hydrolysate was obtained by enzymatic method. Feather waste was purchased from “Lisko broiler” (Country). The feather obtained at the poultry farm was cleaned by washing from animal contamination and products of poultry slaughter, dried at 25±2°C.

The enzyme preparation was proteinase Str. chromogenes s. g. 0832 [13]. Partially purified keratinolytic protease preparation from culture fluid Str. chromogenes s. g. 0832 was obtained by precipitation with 98% isopropanol (in volume ratios of 1:1) in cold at a mixture temperature of 3±0.5°C. Deposition is recommended at pH=7.00. To form a precipitate, the mixture was kept for 20 min. The precipitate was separated on a CN–350 centrifuge (Russia) at 5000 g in plastic cups with a size of 26.5 – 29.5 mm. Then, 100 ml of liquid was placed in each glass. After centrifugation, the precipitate was dried in air prior to the centrifugation at 5000 rpm.

The culture of the microorganism was grown in a thermostat for 12 days at 30±0.5°C on a distilled water–based medium of Chapek with starch composition (g/L): potato starch – 30; NaNO₃ – 2; KH₂PO₄ – 1; MgSO₄ * 7H₂O – 0.5; KCl – 0.05; FeSO₄*7H₂O – 0.1; and agar-agar – 20.

The components of the nutrient medium were dissolved in 1 L of distilled water, heated to complete melting of the agar, poured into test tubes and sterilized for 20 min at 112°C. The Nutrient medium for the cultivation of the microorganism was prepared in accordance with governmental standard 51758–2001. For the cultivation of keratin–splitting protease, a nutrient medium was used, which had the following composition (g/L): potato starch – 40; soy flour – 10; (NH₄)₂SO₄ – 0.65; KH₂PO₄ – 0.45; ZnSO₄ – 0.02; FeSO₄ – 0.01; MnCl₂ – 0.01; CaCO₃ – 3.0; distilled water; pH=6.75. On Wednesday, 0.5% ground chicken feather was introduced as a source of keratin. The pen was previously degreased with chloroform, washed, and dried. Cultivation was carried out under deep conditions in flasks with a volume of 750 ml containing 100 ml of medium at 29±1.0°C on a laboratory rocker with a speed of 240 rpm for 120 h. The nutrient medium was sown with seed material (5vol.%). Actinomycete seed material was prepared on the medium composition: (g/ml): potato starch – 20; soy flour – 20; KH₂PO₄ – 0.5; (NH₄)₂SO₄ – 3.0; NaCl – 2.5; CaCO₃ – 3.0; distilled water; pH=6.60. Cultivation of the seed material was carried out under deep conditions in flasks with a volume of 750 ml containing 100 ml of medium at 28-30°C on a laboratory rocker with a rotation frequency 240 rpm for 48 h.
Pretreatment of pen keratin was performed with sodium sulfide, urea, sodium thioglycolate and sodium tetraborate. Chemical reagents were introduced in an amount of 0.3% by weight. All experiments were carried out with a non-ground pen at a hydromodule of 1:20, at a pressure of 0.15 MPa for 2 h. Enzymatic hydrolysis after keratin treatment was carried out for 6 h with continuous stirring, at a temperature of 40°C and a pH value of 8, the concentration of the enzyme preparation was 3 U/g of protein. The degree of hydrolysis of keratin protein was determined as the ratio of amine nitrogen to the total protein.

The total protein was determined on an automatic turbobrutherm unit (Gerhardt). Amine nitrogen was determined spectrophotometrically using 2,4,6-trinitrobenzenesulfonic acid. The spectrophotometric method of protein determination is based on the ability of aromatic amino acids (tryptophan and tyrosine) to absorb ultraviolet light with a maximum absorption at 280 nm.

Amino acid composition was determined on an automatic amino acid analyzer AAA 339M (Czech Republic) after hydrolysis of 5.7 M HCl in vacuum at 105°C for 12 h.

To determine proteolytic activity (PA), first, a 2% sodium Caseinate solution was prepared in a 0.066 M phosphate buffer (composition) with pH=8.0. Then the test tube was made to 2 ml of the substrate, in the control and 2 ml of enzyme solution and placed at 30°C for 10 min. Then, the test tube was made to 2 ml of the enzyme solution and in the control and 4 ml of 0.3 M trichloroacetic acid. After hydrolysis for 10 min in a test tube was poured in 4 ml of 0.3 M trichloroacetic acid in the control-2 ml 2% solution of sodium Caseinate. Quickly stirred and kept for another 20 minutes at 30°C. Then filtered into dry tubes. From filtrates took 1 ml, added 5 ml 0.5 M solution of Na2CO3, stirred and quickly with continuous stirring poured 1 ml of the working Folin solution. After 30 min, the optical density of solutions was measured on a photoelectrocolorimeter KFC-2 (Russia) at 670 nm in cells with a light-absorbing layer of 10 mm against the control.

For the unit of PA, such an amount of enzyme was taken, which for 1 min at 30° catalyzed the transition to the non-precipitated state of trichloroacetic acid, the amount of sodium Caseinate containing 1 micromole of tyrosine (1 micromole of tyrosine is 0.181 mg).

The content of soluble protein, peptides and amino acids was carried out photocolorimetric. 20 ml of the hydrolysate was taken into glasses with a capacity of 50 ml, 10 ml of 60% trichloroacetic acid was introduced to precipitate non-hydrolyzed proteins. After 20 minutes, the precipitated proteins were filtered on a paper filter, washed 10 ml with 5% trichloroacetic acid. The resulting filtrate was used to determine the amount of peptides and amino acids.

The protein precipitate remaining on the filter was dissolved with 0.1 M NaOH solution, the final volume of 25 ml. Aliquot part of protein solution (10 ml) was used for biuretic reaction: 3 ml 17% KOH, 2 ml 20% CuSO4 were added to 10 ml solution and sample volumes were adjusted to 25 ml 5% KOH. After thorough mixing, the mixture was filtered on a paper filter. The optical density of the supernatant is determined at 590 nm.

1 ml of concentrated H2SO4 and 6 ml of 25% phosphoric acid solution were added to the filtrate obtained after protein precipitation. The precipitate of peptides that fell out was filtered on a paper filter and washed with 15 ml 5% H2SO4. Filtrates were used to determine the amount of free amino acids.

The peptide precipitate was dissolved with 0.1 n NaOH. The final volume is 25 ml. To 10 cm³ solution was added 3 ml 17% KOH, 2 ml 20% CuSO4 and the volume was adjusted to 25 ml 5% KOH. After thorough mixing, the mixture was filtered on a paper filter and used for the estimation of the optical density at 590 nm. The calculation of the content of peptides and proteins was carried out according to the formula (1):

\[ X = \frac{D \cdot 100}{(2.65a)} \% \]  

where, D – optical density; a – the amount of the studied mixture, ml.

From 50 ml filtrate obtained after precipitation of peptides, samples of 2.5 ml were taken, the pH was adjusted to 6.5 – 7.0 by 1 M acetone. Then the volume of samples was adjusted to 5 ml with distilled water. To 1 ml of the neutralized filtrate, 0.5 ml of the ninhydrinated reagent prepared on ethylene glycol monomethyl ether with the addition of tin chloride was added. The ninhydrin reaction was carried out...
in test tubes in a boiling water bath for 20 min. The sample volume was adjusted to 10 ml with distilled water and examined at 570 nm. The content of the sum of amino acids was calculated by the equation (2):

$$X = (D \cdot 27.9) \cdot 100 / a \ , \ %.$$  

In all experiments pH was measured on a potentiometric analyzer.

Biostimulants "Ribav Extra" and hydrolyzed keratin protein were used to treat corn seeds of the variety "Spirit" before sowing in the ground. Seeds were treated with solutions of drugs in concentrations of 0.01, 0.1, 1.0 ml/L of water (control – distilled water), kept for 0.5 h. After processing, the seeds were planted in boxes filled with soil mixture (2 parts compost, 1 part peat) to a depth of 3 – 4 cm. the Plants were grown at a temperature of 25±2°C until the age of 7 days, watering was carried out every other day.

On the third day, the germination energy was determined, on the 7th day – seed germination, and growth characteristics (root and shoot length) were measured.

Seed germination (the number of normally germinated seeds expressed as a percentage of the sample taken for analysis) was determined after germination for 7 days in a thermostat in Petri dishes, the bottom of which was spread with moistened filter paper, at a temperature of 25°C. For germination we used seeds which had a root of at least the length of the seed and a sprout of at least half the length of the seed. Germination energy was determined by the percentage of germinated seeds for 3 days. All the experiments described in the work were carried out three times. Analytical definitions for each sample were made in 3 repetitions. Tables and figures show data where each value is the average of three definitions. Only the results that were reproducible in each experiment are discussed. In the mathematical processing of the results, the student's criterion was used. Differences with the significance level 5% were considered to be reliable.

3. Results and discussion

Under experimental conditions, it was found that the treatment of corn seeds with biostimulator Ribav Extra at a concentration of 0.01 ml/L did not lead to noticeable improvements in the estimated parameters (table.1). The germination energy was at the control level, increasing the biostimulator concentration to 0.1 ml/L increased the seed germination energy by 12%. A further increase in the concentration of the drug to 1.0 ml/L led to a decrease of this indicator by 5%.

Almost the same dynamics is observed in the evaluation of seed germination (table.1). Treatment with the drug at a concentration of 1.0 ml/L reduced germination by 11%, the concentration of 0.01 ml/L gave almost no positive results. Treatment of Ribav Extra at a concentration of 0.1 ml/L increased seed germination by 5% compared to the control.

The effect of Ribav Extra on root length in concentrations of 0.01 and 1.0 ml/L was negative and led to a decrease of 3% and 17.9%, respectively. Treatment of seeds with the preparation at a concentration of 0.01 ml/L on the contrary increased the length of the root by 5%.

When assessing the length of the shoot of plants, Ribav Extra treatment in all concentrations gave a positive result, but the concentration of 0.01 ml/L was again the best. The increase in this case was 26.7%.

When processing seeds biostimulator of keratin pen in all cases, there is a positive trend. It is worth noting that at a concentration of 0.1 ml/L hydrolysate from keratin pen as well as Ribav Extra gave better results. Thus, the germination energy was higher than the control samples by 18%, germination increased by 10% compared to the control and by 5% compared to Ribav Extra. The length of the root was greater than control by 17.9%, the length of the shoot – by 49%. Even with a minimum concentration of hydrolysate from keratin pen (0.01 ml/L) germination energy increased by 14%, germination – by 7%, root length – by 11%, shoot length – by 33%. The increase in the concentration of the biostimulator from keratin pen to 1.0 ml/L, unlike Ribav Extra, did not have a negative effect, and it also exceeded the control parameters: energy growth – by 9%, germination – by 5%, shoot length – by 16%. The length of the root of the plants did not increase and was at the level with the control.
Table 1. Effect of biostimulants on the germination of corn seeds and the length of the axial organs of plants.

| Option experience's | Drug concentration, ml/L | Germination energy, % | Germination, % | Length, mm |
|---------------------|--------------------------|-----------------------|----------------|------------|
| Control             | 0                        | 75±1.7                | 85±1.5         | 95±2.1     | 45±4.6     |
| Ribav Extra         | 1.0                      | 70±2.7                | 74±2.6         | 78±6.5     | 51±3.6     |
| Ribav Extra         | 0.1                      | 87±1.7                | 90±1.6         | 100±6.3    | 57±3.2     |
| Ribav Extra         | 0.01                     | 75±1.5                | 86±1.6         | 92±6.0     | 53±3.0     |
| Hydrolysate of keratin feather | 1.0 | 84±1.8 | 90±1.7 | 95±6.2 | 52±3.2 |
| Hydrolysate of keratin feather | 0.1 | 93±1.7 | 95±1.6 | 112±6.0 | 67±2.7 |
| Hydrolysate of keratin feather | 0.01 | 89±1.8 | 92±1.7 | 105±5.7 | 60±2.4 |

It can be noted that all the estimated parameters when treated with keratin hydrolysate pen gave higher results than the treatment of Ribav Extra. Many authors note [14] that biostimulants of animal origin reduce the effect of temperature stress on plants. It was found that biostimulants of animal origin (in particular pepton) increase the stress resistance of plants, enhance the development of roots [15]. Treatment of plant roots with hydrolyzed proteins increases nitrogen uptake by increasing the activity of reductase and glutamine synthetase [16]. It should be noted that the origin of biostimulants is different: vegetable (Ribav Extra) and animal (keratin hydrolysate). The active substance in the biostimulator of plant origin are L-alanine and L-glutamic acid. The hydrolysate from pen keratin is represented by a set of 18 amino acids (figure 1).

Figure 1. Amino acid composition of keratin feather.

Based on the results of the study, it can be assumed that both stimulants have common functional compounds that activate metabolic processes in plant cells. However, the biostimulator from feather keratin has a more balanced amino acid composition. It contains all essential amino acids, hydrolysate high content of glutamic acid, Proline, leucine and valine. Glutamic acid stimulates plant growth, activates seed germination, since it is a precursor for the synthesis of other amino acids and chlorophyll. Proline-increases the immunity of plants in stressful situations, promotes the accumulation of nitrogen, as well as glutamic acid improves the efficiency of photosynthesis and increases the content of chlorophyll, regulates water exchange in plants. It is well known that Proline under water stress prevents
dehydration of plants. This, in turn, stabilizes nitrogen metabolism, improves the hormonal balance of the plant. Leucine and valine increase plant resistance to high temperatures [17].

In our study, after treatment of seeds with a biostimulator from keratin hydrolysate at a concentration of 0.1 ml / L, the length of the plant root increased by 18% compared to the control (seeds not treated with a biostimulator). Treatment of seeds with the drug "Ribav Extra" in the same concentration gave an increase in the length of the roots of plants by only 5%. Perhaps, in our case, the presence of tryptophan and phenylalanine in the hydrolysate (aromatic amino acids) stimulate the synthesis of auxin, as indicated by the studies of some scientists [18-20]. This may explain such an intensive increase in root formation in plants after treatment with a biostimulator from keratin hydrolysate pen.

Comparison of the length of plant shoots showed that the effectiveness of the resulting preparation from keratin pen is higher than the existing analogue by 23% and 48% compared to the control. The apparently richer amino acid composition of the biostimulator from keratin hydrolysate (18 amino acids) allows plants to obtain the molecule necessary for the synthesis of the desired substance at the moment, and not to waste additional time and energy on nutrition.

We managed to achieve such a deep hydrolysis of pen keratin thanks to enzymatic hydrolysis with an enzyme preparation from actinomycete Str. chromogenes s.g. 0832.

Enzymatic hydrolysis with Streptomyces chromogenes s.g. 0832 allowed to obtain a product with high biological value. The hydrolysate contains all the essential amino acids. Glutamic acid and Proline were found to be particularly abundant in the hydrolysate. The total amount of amino acids was 99.57 wt%, the amount of essential amino acids was 42.66 wt%.

It is generally recognized that the keratinolytic process includes two stages: sulfitolysis and proteolysis [21,22]. At the stage of sulfitolysis, the protein is prepared for enzymatic attack, the stability of the spatial structure of the protein molecule decreases due to the rupture of disulfide bonds.

Pretreatment of feather keratin with sodium sulfide and urea in our case did not give a high effect of destruction of the protein (table.2). Further fermentation increased the solubility of the pen by only 3.8% after treatment with sodium sulfide and 7.2% after exposure to urea. Perhaps, such low values of soluble keratin are associated with a low dose of introduced substances (0.3%). Apparently, this concentration of sodium sulfide and urea is not sufficient to prepare the protein for enzymatic hydrolysis. In similar works, the authors contribute at least 2% of the chemical reagent to prepare keratin for fermentation [21]. In our studies, we deliberately did not increase the concentration of reagents, as this could affect the destruction of amino acids. Treatment of keratin with sodium thioglycolate increases the yield of soluble protein 5–10 times in comparison with urea and sodium sulfide. In the presence of sodium tetraborate, pen keratin loses its stability as much as possible. Subsequent fermentation allowed to obtain almost soluble protein.

| Chemical reagent       | Degree of hydrolysis% before fermentation | Degree of hydrolysis% after fermentation |
|------------------------|------------------------------------------|----------------------------------------|
| Sodium sulphide        | 1.4±0.7                                  | 5.2±0.8                                |
| Urea                   | 2.8±0.5                                  | 10.0±0.7                               |
| Sodium thioglycolate   | 3.7±0.5                                  | 50.0±0.9                               |
| Sodium tetraborate     | 5.0±0.4                                  | 80.0±0.7                               |

Apparently, treatment with sodium tetraborate promotes deeper deployment of peptide chains of the keratin molecule. As a result, those reactive groups of the native protein that were previously unavailable for the action of the enzyme become available and hydrolysis proceeds more fully. The obtained data correlate with the results of a number of studies [23, 24], where it is confirmed that the degree of degradation of keratin is enhanced by pretreatment with appropriate chemical reagents. It was found that the presence of 2% reagent, sodium thioglycolate contributed to the enzymatic decomposition of keratin in the first 2 h and was almost completely completed by 6 h of hydrolysis [21]. The results of
our studies presented in table 3 also confirm that the initial attack of keratin disulfide bonds by sodium tetraborate plays an important role for the complete decomposition of the native protein. Basically, the end products of keratin hydrolysis are free amino acids, and after 1 h after fermentation, their content was 82.2%, and by 6 h – 89.0%. Amino acid composition of the resulting product (figure 1) indicates the predominant hydrolysis of polarized sections of the polypeptide chain of the keratin macromolecule. According to the total concentration of amino acids, the first place is occupied by amino acids with a carboxyl group (aspartic and glutamic) and with an amino group (arginine, histidine and leucine) the Presence of cystine indicates partial hydrolysis of disulfide bonds in keratin.

Table 3. Dynamics of accumulation of products of enzymatic hydrolysis of keratin protein.

| Duration of hydrolysis, h | soluble protein | peptides | amino acid |
|--------------------------|----------------|---------|------------|
| 0                        | 4.2±1.5        | 1.4±0.4 | 0          |
| 1                        | 4.5±1.3        | 1.5±0.3 | 82.2±1.3   |
| 2                        | 4.7±1.3        | 1.8±0.5 | 87.1±1.2   |
| 3                        | 4.7±1.2        | 2.2±0.3 | 88.4±1.2   |
| 4                        | 6.1±1.4        | 2.4±0.3 | 88.6±1.4   |
| 5                        | 5.1±1.2        | 3.2±0.2 | 88.9±1.3   |
| 6                        | 5.1±1.2        | 3.6±0.3 | 89.0±1.3   |

The experimental data on the dynamics of keratin hydrolysis presented in table 3 show that intensive cleavage of keratin-containing raw materials by the enzyme preparation *Str. chromogenes s. g. 0832* occurs in the first two hours and reaches a maximum value of 90-92% by 4-6 h.

4. Conclusion

Our results confirm studies on the positive effect of animal biostimulants on plants. The qualitative and quantitative composition of the protein affects the effectiveness of the biostimulator. The same concentration can increase the growth of some organs and inhibit others. So, the concentration of biostimulants considered 1.0 ml/L, inhibits the growth of the root and increases the growth of the shoot. At the concentration of Ribav Extra 0.01 ml/L only the length of the shoot increases. Thanks to the selected conditions of keratin treatment, it was possible to obtain a product with a high content of amino acids that affect the germination and germination of corn seeds. The high content of free amino acids in the final product stimulates root formation and development of plants.

The content of free amino acids in it is 82.2%. The high content of free amino acids in the final product stimulates root formation and development of plants. Tryptophan and phenylalanine (aromatic amino acids) stimulate auxin synthesis. By accelerating cell division, auxin promotes apical dominance, stimulates root formation, and increases nitrogen uptake. This explains such an intensive increase in the axial organs of plants after treatment with a biostimulator of keratin hydrolysate pen. The length of the root increased by 1.2 times, the length of the shoot – by 1.5 times in comparison with the control.

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