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Properties of novel protease of *Streptomyces* sp. required for fuel ethanol production

New protease isolated from Streptomyces sp. 12 was studied. The maximal specific proteolytic activity of purified protease was 538 U·mg of protein^{-1}, Mr is about 35.6 kDa and optimal conditions of hydrolysis give effect at pH 8.0 and temperature 50 °C. Study of the substrate specificity permits to use enzyme in fuel production

Today protease attracted the attention of researchers because they perform a wide variety of complex physiological functions. Their importance in the implementation of metabolic and regulatory functions is confirmed by the fact that they are present in all forms of living organisms. Intracellular proteases play a critical role in the regulation of metabolism, while extracellular proteases catalyze the hydrolysis of proteins that are found in the extracellular environment and transform them into a form that can easily penetrate inside the bacterial cell. They can be also used in fuel production from corn starch granules. It's known that in fuel ethanol production, energy used in the cooking step is 10 to 20 % of the total energy content. The granular starch hydrolysis (GSH) process is claimed to save energy and reduce capital cost by eliminating cooking. Corn starch granules are embedded in the protein matrix. Bonds holding the matrix protein together can be lost by treatments with alkali or reducing agents, such as mercaptoethanol or sulfite. The protein matrix can be digested by proteases more rapidly than zeins. The proteases have an effect of endosperm structure [1]. Starch granules in corn are encapsulated by endosperm-associated proteins in a protein matrix. Proteases degrade the protein matrix surrounding the starch granules and help release starch that increased mash specific gravity and improved germ recovery in the enzymatic wet-milling process. In addition, proteases increase fermentation rates by hydrolyzing proteins into free amino nitrogen (FAN) [2]. FAN produced by a protease could be substituted for an exogenous nitrogen source (urea, inorganic ammonium ions, or amino acids) needed by yeast during fermentation. Adding proteases could reduce granular starch hydrolyzing enzymes usage and eliminate addition of other yeast nutrients.

Among the producers of protease the soil microorganisms occupy an important place. They belong to different taxonomic groups, but most of them are represented by micromicetes and streptomycetes [3]. Literary sources indicate that streptomycetes have the potential to decompose various natural polymers such as chitin and pectin. The interest of these producers is also caused by that they are able to produce proteolytic enzymes with different substrate specificity, which was shown [4], are safe for use in the manufacture of food and drugs (FDA-approved or GRAS).

Previously [5] at the Department of Biochemistry of microorganisms of Zabolotny Institute of Microbiology and Virology of NASU as a result of screening 45 strains of *Streptomyces* sp. isolated from the rhizosphere of various plants were

selected *Streptomyces* sp. 1 2, which have the highest proteolytic activity.

The aim of this study was an optimization of the cultivation conditions of *Streptomyces* sp. 12, which provide a maximum synthesis of proteases to obtain a purified preparation of the protease, to investigate its substrate specificity, physico-chemical properties and functional groups of the active site.

Optimization of cultivation conditions using single-factor experiments were carried out on the base medium of the following composition (g/l): K_2HPO_4 – 1; $MgSO_4 \cdot 7H_2O$ – 1; NaCl – 1; $(NH_4)_2SO_4$ – 2; $CaCO_3$ – 2; starch – 10; microelements salt solution ($FeSO_4 \cdot 7H_2O$ – 1, $MnCl_2 \cdot 4H_2O$ – 1, $ZnSO_4 \cdot 7H_2O$ – 1) – 1 ml. Total caseinolytic (proteolytic) activity was quantitatively determined on tyrosine, which is formed by hydrolysis of casein under the action of the investigated enzyme. Collagenase activity colorimetrically is determined by ninhydrin. The number of units of activity equals to the amount of μmol L-leucine, which is freed from collagen after 5 hours at 37 °C. To isolate and purify of the enzyme it is used precipitation by $(NH_4)_2SO_4$ (90% saturation), chromatography on neutral and charged TSK-gels. The homogeneity of the enzyme preparation was determined in native system by gel-filtration on Sepharose 6B ("Pharmacia", Sweden). Investigation of the influence of pH and temperature of environment on the activity of purified protease of *Streptomyces* sp. 12 was performed in the temperature range from 6 to 80 °C and a pH of 5.0 to 11.0.

It is established that optimal sources of carbon, nitrogen and minerals in the nutritious medium are corn meal, $(NH_4)_2SO_4$ and $CaCl_2$ respectively. The growth of *Streptomyces* sp. 12 was carried out in submerged conditions during 3 days in 200 ml of the optimized nutritious medium at initial value of pH 7.0, temperature 37° C, 220 rpm. The specific proteolytic activity was 62.7 U·mg of protein^{-1}, which is 4.8-fold higher than in the control (base medium). Similar to our results there are the data of study of proteases of other actinomycetes *Saccharomonospora viridis* SJ-21 [6].

Using fractionation by sulphate ammonium, gel-filtration and ion-exchange chromatography on TSK-gels – Toyopearl HW-55, DEAE 650(M) and Sepharose 6B, *Streptomyces* sp. 12 protease with specific activity 538 U·mg of protein^{-1} and yields 35.8 % was isolated. With the use of proteins-markers have been shown (Fig. 1), that the molecular weight of investigated protease is about 35.6 kDa. The literature often describes the extracellular proteases of streptomycetes with a molecular weight of 14 and 70 kDa.

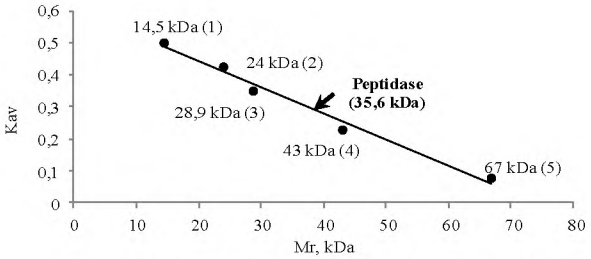

Fig. 1. Determination of molecular weight of *Streptomyces* sp. 12 protease in the native conditions (calibration plot): Kav – distribution ratio; proteins-markers: lysozyme (1), trypsin (2), proteinase K (3), peroxidase (4), bovine serum albumin (5)

It is shown that enzyme displays highest activity toward collagen and less activity toward casein, albumin and gelatin (fig. 2). This may be due to the affinity of protease to Gly or Pro, which are prevalent in the structure of collagen molecules.

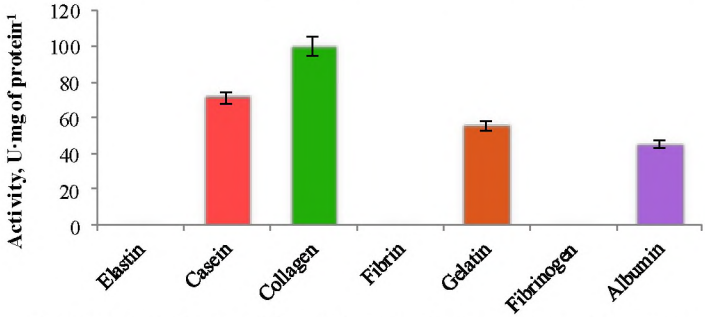

Fig. 2. Hydrolysis of protein substrates by *Streptomyces* sp. 12 protease

Conducted by us studies of the influence of group-specific chemical reagents (0.01 M) showed that tested enzyme is metalloprotease with optimal conditions of action on collagen at pH 8.0 and temperature 50 °C.

So far as the major corn zein amino acids are Pro, Glu and Ala give a possibility to suppose a perspectivity of usage of *Streptomyces* sp. 12 protease in fuel ethanol.

Conclusions

Thus, studied protease of *Streptomyces* sp. 12 with high collagenase activity which is alkaline and thermo stable enzyme may be suitable to use for fuel ethanol production to degrade the protein matrix and help release starch.

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