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Novel milk-clotting enzyme produced by *Coprinus lagopides* basidial mushroom

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ABSTRACT

Submerged cultured higher basidiomycetes were screened for milk-clotting activity. The native liquid of *Coprinus lagopides* demonstrated high milk-clotting activity, while having relatively low general proteolytic activity. Optimization of growth media composition and ultrafiltration of the native liquid allows increasing the ratio of milk-clotting and proteolytic activities. The Enzyme was purified and characterized.

1. Introduction

1.1. The selection of the milk-clotting enzyme is essential for making

Successful choice of cheese production technological process. For millennia the rennet (rennin, chymosin), produced in the abomasum (part of the stomach) of young ruminant animals, was used for milk coagulation in cheese-making. The rennet performs the same function in the organism of mammals, as in the cheese-making – it clots milk, starting the process of its digestion.

The quality of enzymes used for cheese making, influences both, the process of the formation of milk curds, and also the process of the biotransformation of the components of milk into the compounds, which finally determine the organoleptic properties of the end dairy product. In other words, the basis of the quality of future cheese is laid at the early stage of milk coagulation.

Because of inexpediency of the slaughtering of young animals of the milk life cycle, proteolytic enzymes of microbial origin, with the properties close to those of the rennet have become widely used for cheese making in recent decades (Bukhalo, 1988, chap. 1; Gudkov, 2004, chap. 2). Unfortunately, due to concomitant proteolytic activity obtained clots often have a bitter taste (He et al., 2011; Raposo & Domingos, 2008).

The replacement of the expensive rennet by microbial proteases of specific action is economically advantageous, promising and is always urgent (Gudkov, 2004, chap. 2; Teply, Mashek, & Havlova, 1980, chap. 3).

Requirements for the substitutes of the rennet are strict and specific their enzymatic properties must be maximally close to those of the enzyme accepted as the standard natural rennin, i.e. along with high milk-clotting activity they must possess the insignificant general proteolytic activity, which would result in unspecific proteolysis of casein (Emmons, 1990; Preetha & Boopathy, 1994).

The success of screening for milk-clotting enzymes in different groups of the organisms of different levels of organization is complicated by the aforementioned rigid requirements – the high ratio of milk-clotting activity to general proteolytic activity. Highly active proteases with the same action as that of rennet were discovered in the higher basidiomycetes, and some of them have found practical application. The analysis of literature references shows that the continuation of search in this group of macromycetes can prove to be successful. The aim of this study was screening for the new efficient producer of milk-clotting enzyme among strains of basidiomycetes and development of the production method.

2. Materials and methods

2.1. Materials

The subject of our study were cultures of strains of the higher basidia fungi – *Trametes suaveolens* (L.:Fr.) Fr., *Trametes ochracea*

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(Pers.) Gilb. & Ryvarden *Fomes fomentarius* (L.:Fr.) Fr., *Ganoderma lucidum* (Leyss.: Fr.) P.Karst., *Bjerkandera adusta* (Fr.) Karst., *Grifola frondosa* (Dicks.: Fr.) Gray, *Flammulina velutipes* (Fr.) P.Karst., *Panus conchatus* (Bull.: Fr.) Fr., *Coprinus lagopides* P.Karst., *Pleurotus ostreatus* (Jacq.:Fr.) Quel. Their wild-grown fruit bodies were collected in different geographical regions, including suburbs of Saint Petersburg, and they were introduced into culture.

Milk-clotting enzyme preparation of animal origin obtained from All-Russian Institute of Butter and Cheese making (Uglich city), which is an industrial standard preparation, was used as a reference sample.

2.2. Culturing

Initial cultures were cultivated under submerged conditions in flasks at a temperature of 28 °C (*F. velutipes* at 20 °C) for 7 days on the rotary shaker (IR-1LT, Labtech, Moscow, Russia) using semi – synthetic and natural media. Semi-synthetic medium contained (g/L): glucose – 10; peptone – 2.5; KH_2P04 – 0.6; K_2HPO4 – 0.4; CaCl2 – 0.05; NaCl – 0.5; yeast extract –2.0; pH of the media before sterilization – 5.8–6.0. Also the influence of elevated levels of glucose (40 g/L) and peptone (10 g/L) on the enzyme production were studied.

Natural medium consisted of the beer wort ("Capitan" brewery, St. Petersburg, Russia) diluted to the concentration of sugar of 40 g/L with addition of soybean flour – 10 g/L, pH of media before sterilization was 5.8–6.0.

After separation of mycelium by filtration from culture broth, native liquid was used for farther study.

2.3. Enzymatic assay

2.3.1. Milk-clotting activity

The level of the milk-clotting activity (MCA) was determined according to the method of Kawai–Mukai (Kawai & Mukai, 1970). The method for determination of the milk-clotting activity (MCA) is based on the determination of time, necessary for clot formation. Fresh commercial milk (25 g/L fat) with addition of $CaCl_2$ (150 mg/L), pH 6.0 was used as a substrate. One unit of MCA was defined as the amount of enzyme, required to clot 100 ml of milk within 40 min at 35 °C (Teply et al., 1980, chap. 3):

$$\mathrm{MCA} = 40 \times 100 \times \mathrm{C}/2\mathrm{PUnits/ml}$$

were C – dilution coefficient of native liquid or enzyme preparation,

P – period of time (min), during which a dense milk curd forms as a result of adding enzyme or native liquid,
40 – average time (min), during which 100 ml of milk is curdled,
2 – volume (ml) of added enzyme or native liquid.

2.3.2. General proteolytic activity

The level of the total proteolytic activity was determined according to the Russian state standard (GOST) method (GOST «20264.2 – 88»), based on hydrolyses of casein proteins. This method for determination of the general proteolytic activity (PA) is based on hydrolysis of the proteins of casein by the studied preparation and subsequent inactivation of enzyme and precipitation of the non-hydrolyzed protein by trichloroacetic acid (TCA). One unit of proteolytic activity was defined as the amount of enzyme, required to convert casein into non-TCA-precipitable form within 1 min at 30 °C in the quantity, which corresponds to 1 μmol of tyrosine. The activity is expressed in U/mg of protein.

2.3.3. Kinetic parameters

Kinetics of enzymatic activity of obtained milk-clotting preparations was characterized through maximum rate of proteolytic reaction and Michaelis constant. These kinetic parameters were determined based on linearization of Michaelis–Menten equation in the inverse values:

$$1/v = \{\mathrm{Km/Vmax}^*[\mathrm{S}]\} + 1/\mathrm{Vmax},$$

were v – reaction rate,

Vmax – maximum reaction rate,
Km – Michaelis constant,
So – substrate concentration.

2.3.4. Determination of optimum temperature and pH for enzymatic activity

Evaluation of the influence of temperature on the activity of milk-clotting enzyme preparation, was done by performing enzymatic assay using the fore mentioned method of Kawai–Mukai at different temperatures (from 25 to 45 °C) at pH 6.0.

The influence of pH level on the enzymatic activity was determined by the same method at different pH values (from 6.0 to 8.5) at the same temperature of 35 °C.

2.4. Determination of protein concentration

The content of protein in the studied solutions was determined by the Lowry's method (Lowry, Rosebrough, Farr, & Randall, 1951).

2.5. Enzyme purification

An ultrafiltration method (membranes "MIFIL-PA-20" (IPhOCh, Minsk, Belarus) with a limit of nominal molecular weight retention of membranes of 20 kDa) was used to purify and concentrate enzymes.

Ultrafiltrate was lyophilized to obtain powder preparation. Dried enzyme preparation for further studies was used dissolved in 0.01 mol/L Na-phosphate buffer (pH 6.8). When determining the kinetic constants, the concentration of milk-clotting enzyme preparation in samples with different concentrations of the substrate (milk) was constant and was 27 mg/ml, corresponding to 50 U/ml.

2.6. Determination of molecular weight

The molecular weight of enzyme preparation was tentatively evaluate by gel-chromatography. 0.5 ml samples of mushroom milk-clotting enzyme were applied to the chromatographic column (40 cm height, 1.5 cm diameter, "Lenchrom", St. Petersburg, Russia) filled with Sephadex G-75 gel. Elution was conducted by physiological saline (8.5 g/L NaCl).

The solution, emerging from the column, was colleted as 2.8 ml fractions, and their optical density was measured (SF 46 spectrophotometer, "LOMO", Russia), at the wavelength of 280 nm.

Fractions with the highest values of optical density were used for evaluation of enzymatic activity. The protein content of each fraction was estimated by the method of Lowry and by adsorption coefficient at the wavelength of 280 nm.

Standard proteins with known molecular weight – bovine serum albumin (68 kDa), hemoglobin (64 kDa), lactalbumin (36 kDa) deoxyribonuclease (31 kDa), trypsin (23.8 kDa), as well as ribonuclease (13.7 kDa) were used for calibration (Mikesh, 1982, part 1, chap. 6).

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