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EXPERIMENTAL ARTICLES

Extracellular L-Glutamate Oxidase of *Streptomyces* sp. Z-11-6: Obtainment and Properties

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Abstract—Mutagenesis induced with nitrous acid and subsequent selection allowed a genetically stable mutant strain, *Streptomyces* sp. Z-11-6, to be obtained, whose L-glutamate oxidase activity was 40-fold higher than that of the original natural isolate and was as great as 1.6–1.8 units/ml of culture liquid. A procedure for the isolation and purification of the enzyme was developed; the biochemical properties of the enzyme were studied. Out of 20 amino acids tested (including D-glutamate), the glutamate oxidase from *Streptomyces* sp. Z-11-6 was active only with L-glutamate. This allows the concentration of L-glutamate to be determined in the presence of other amino acids. Calcium chloride at a concentration of 0.1–0.5% promoted the secretion of the extracellular glutamate oxidase.

Key words: L-Glutamate oxidase, isolation and purification, biochemical properties.

Extracellular glutamate oxidase (L-glutamate: O_2 oxidoreductase (deaminase), EC 1.4.3.11) is an enzyme that specifically catalyzes the oxidative deamination of L-glutamate in the presence of water and oxygen with the formation of α-ketoglutarate, ammonia, and hydrogen peroxide:

$$L\text{-glutamate} + O_2 + H_2O \xrightarrow{\text{glutamate oxidese}} \alpha\text{-ketoglutamate} + NH_3 + H_2O_2.$$

The hydrogen peroxide formed in this reaction can easily be detected by the chromogenic peroxidase reaction. Therefore, glutamate oxidase can be used as an analytic reagent and as a basis for developing biosensors for the determination of L-glutamate, L-glutamine, ammonia, and creatinine; these biosensors can be used in analytical chemistry for quantitative and qualitative assays of enzymatic processes, in the food industry to assess the quality of foodstuffs [1, 2], and in clinical biochemistry for the determination of glutamate–pyruvate transaminase and glutamate–oxalacetate transaminase in biological fluids, which makes the early diagnosis of heart and liver diseases possible [3].

Earlier [4], by means of mutagenesis induced with nitrous acid and subsequent selection, we managed to obtain a genetically stable producer of L-glutamate oxidase, strain *Streptomyces* sp. Z-11-6.

The aim of the present work was to develop a procedure for the isolation and purification of this extracellular enzyme and to study its biochemical properties and the effect of calcium ions on its secretion.

MATERIALS AND METHODS

Producer strain and cultivation conditions. The mutant strain *Streptomyces* sp. Z-11-6 [4] was used as the producer of extracellular glutamate oxidase.

The strain was maintained on oat agar. In the experiments, it was grown in liquid medium containing glucose and corn extract [4] on a shaker (190 rpm) at 26°C.

Determination of glutamate oxidase activity was carried out on the basis of the chromogenic peroxidase reaction [4]. The amount of the enzyme catalyzing the formation of 1 μmol of hydrogen peroxide per minute at 37°C was taken as the activity unit.

Investigation of the effect of calcium ions on glutamate oxidase secretion. The inoculum was grown overnight at 26°C on a shaker (190 rpm) and transferred (5 vol %) into 750-ml shake flasks containing 100 ml of the medium. Immediately before inoculation, $CaCl_2$ was introduced into the flasks to a final concentration of 0.1–0.5%. $CaCl_2$-free medium was used as a control. Cultivation was performed at 26°C on a shaker (190 rpm). Samples were withdrawn every four hours to determine the enzyme activity and biomass.

Biomass determination. Biomass was determined as the weight of dry mycelium washed with 1 N HCl and then two times with distilled water. Filters with washed mycelium were dried to a constant weight at 80°C and stored in a desiccator with silica gel prior to weighing.

Protein determination. Protein in the enzyme preparations was determined by the method of Lowry *et al.* [5].

Analytical grade reagents purchased from Reakhim (Russia) were used in this work.

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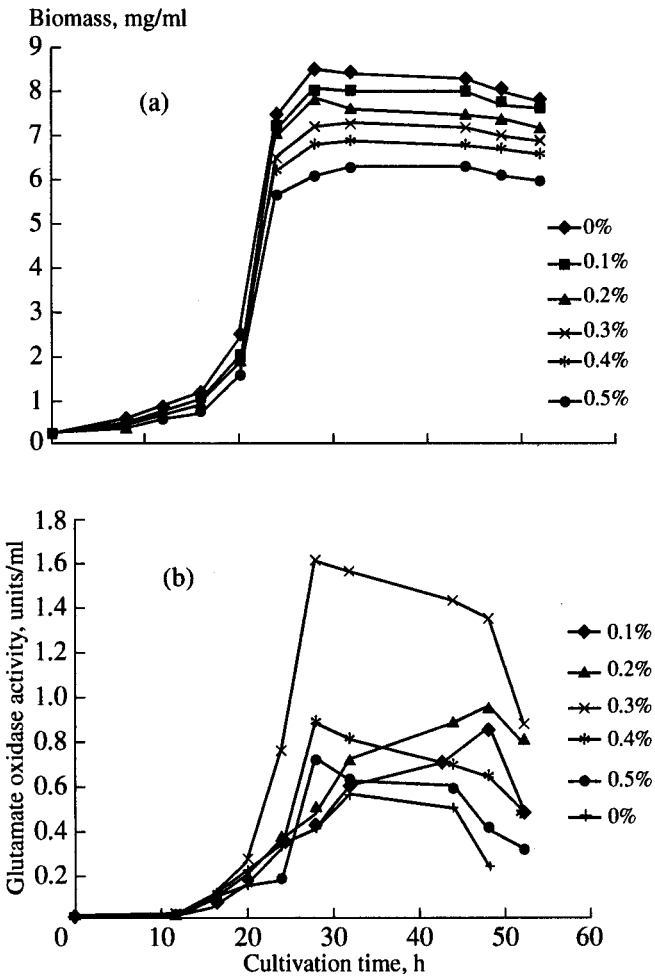

Fig. 1. Effect of calcium ions on the (a) growth and (b) glutamate oxidase activity of *Streptomyces* sp. Z-11-6.

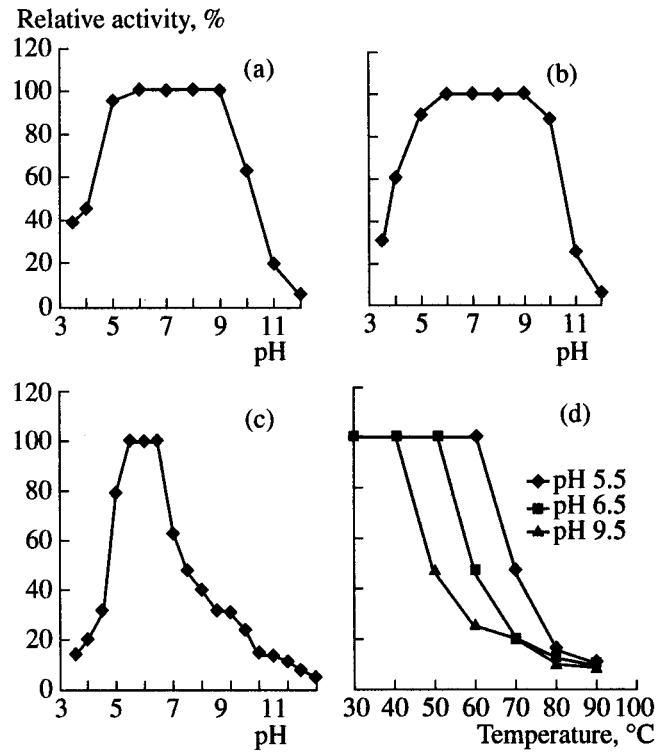

Fig. 2. Stability of extracellular glutamate oxidase of *Streptomyces* sp. Z-11-6 as a function of pH and temperature: residual activity of the enzyme after incubation at various pH values at (a) 37°C for 60 min, (b) 45°C for 15 min, (c) 60°C for 15 min or (d) at various temperatures from 30 to 90°C.

RESULTS AND DISCUSSION

Isolation and Purification of the Enzyme

A procedure for the isolation and purification of extracellular glutamate oxidase involved filtration of the culture liquid of *Streptomyces* sp. Z-11-6 through a dense cloth and 10-fold concentration of the filtrate on an AR-0.1[0.2 mPa] apparatus (Biotest, Kirishi, Russia). Proteins were precipitated from the concentrated filtrate with ammonium sulfate (50% of the saturating concentration). The precipitate was collected by centrifugation (8000 g, 15 min), dissolved in 0.05 M K–phosphate buffer (pH 7.0) containing 0.6 M NaCl, and applied to a 2.0 × 25 cm column with DEAE cellulose equilibrated with 0.05 M K–phosphate buffer (pH 7.0). The active fractions collected were dialyzed against 0.005 M K–phosphate buffer (pH 7.0) for 20 h. The precipitate formed in the course of dialysis was removed by centrifugation (8000 g, 10 min), and the supernatant was applied to a 2.0 × 25 cm column with DEAE cellulose equilibrated with 0.05 M K–phosphate buffer (pH 7.0). The active fractions collected were lyophilized. The purity of the enzyme was checked by nondenaturing electrophoresis in 4–12% PAAG gradient in the presence of HMW standards (Pharmacia, Sweden) [6]. The enzyme thus purified had a specific activity of about 25 units/mg protein; the enzyme yield was about 30%. According to data available in the literature, partially purified glutamate oxidases from other *Streptomyces* strains had a specific activity of 10–50 units/mg protein; the enzyme yield was 18–20% [7, 8].

The Effect of Calcium Ions

Calcium ions have been reported to produce a positive effect on the secretion of a number of extracellular enzymes [9]. In our experiments, the addition of 0.1–0.5% $CaCl_2$ to the medium stimulated the secretion of extracellular glutamate oxidase by *Streptomyces* sp. Z-11-6. The presence of calcium ions decreased the biomass yield; the effect was concentration-dependent (Fig. 1a). Thus, after 28 h of cultivation, the dry mycelium weight in the calcium-free medium was 40% higher than in medium with 0.5% $CaCl_2$. The growth rate was also higher in calcium-free medium or in media with low $CaCl_2$ concentrations (0.1% and 0.2%): in these media, the maximum biomass yield was attained after 28 h of cultivation, whereas in media with

0.3–0.5% $CaCl_2$, the maximum biomass was accumulated after 44 h. Glutamate oxidase activity in calcium-free medium and in media with low calcium concentration attained its maximum after 48 h of cultivation and was two times lower than the maximum activity observed in medium with 0.3% $CaCl_2$ after 28 h of cultivation (1.6–1.8 units/ml culture liquid, Fig. 1b).

Thus, we found that the addition of calcium ions to the medium increased the yield of glutamate oxidase and shortened the cultivation time; this would decrease the cost of industrial production.

Biochemical Properties of the L-Glutamate Oxidase Studied

Substrate specificity. Most of the glutamate oxidases studied so far are characterized by a low substrate specificity [7, 10, 11].

The enzyme obtained in this work exhibited a high specificity toward L-glutamate: out of 20 amino acids tested (including D-glutamate), the glutamate oxidase of *Streptomyces* sp. Z-11-6 was highly active only with L-glutamate; this allows the concentration of L-glutamate to be determined in the presence of other amino acids. The K_M value of the enzyme studied for L-glutamate, determined at pH 7.4 using the Lineweaver–Burk plot, equaled 0.34 mM, whereas the affinities of glutamate oxidases from other microbial strains to L-glutamate were reported to be characterized by K_M values of 1.1–3.8 mM at pH 7.4 [7, 8, 10–12].

pH optimum. The activity of the enzyme at various pH values was determined at 30°C; the substrate was L-glutamate in 0.2 M acetate buffer (pH 3.5–6.0), 0.2 M K–phosphate buffer (pH 6.0–8.5), or 0.2 M glycine–NaOH buffer (pH 8.5–11.5).

According to data available in the literature, the pH optimum of the glutamate oxidases studied is in the pH range of either 5.0–7.5 [8, 10, 12] or 7.0–8.5 [7]. The enzyme investigated in the present work has a broad pH optimum of 6.5–9.5.

pH stability. The enzyme studied was incubated at pH values from 3.5 to 11.5 at 37°C for 60 min, at 45°C for 15 min, or at 60°C for 15 min. Then, the enzyme was rapidly cooled, and glutamate oxidase activity was measured. Incubation at 37°C for 60 min did not inactivate the enzyme at pH 5.5–9.5 (Fig. 2a), whereas glutamate oxidases from other microbial strains withstand such a heat treatment at pH 4.5–8.0 [7, 11, 12]. Incubation at 45°C for 15 min did not inactivate the enzyme at pH 5.5–9.5 (Fig. 2b). Incubation at 60°C for 15 min did not inactivate the enzyme at pH 5.5–6.5 (Fig. 2c).

Temperature range for the enzyme activity. A reaction mixture containing 10 mM L-glutamate was incubated at temperatures from 20 to 90°C for 20 min. The enzyme activity was recorded within a temperature range of 20–65°C, with an optimum at 40°C.

Effect of metal ions, inhibitors, and chelating agents on the activity of the extracellular L-glutamate oxidase of *Streptomyces* sp. Z-11-6

Additives, 1 mM	Relative activity, %	Additives, 1 mM	Relative activity, %
None (control)	100	Li_2SO_4	95.7
KCl	93.5	$ZnSO_4$	93.8
NaCl	95.7	$MnSO_4$	95.7
KI	103.6	$CoSO_4$	100
NaF	102.6	$Al_2(SO_4)_3$	93.8
$CaCl_2$	101.2	EDTA[1]	107.8
$CuCl_2$	101.2	NEM[2]	94.4
$BaCl_2$	105.6	PCMB[3]	82
$NiCl_2$	104.5	KSCN	90
$StCl_2$	97.2	Na cholate	95.4
$CoCl_2$	98.2	NaN_3	100
$FeCl_3$	92.8	DDTC[4]	98.6

[1] EDTA, ethylenediaminetetraacetic acid.
[2] NEM, *N*-ethylmaleimide.
[3] PCMB, *p*-chloromercuribenzoate.
[4] DDTC, sodium diethyldithiocarbamate.

Heat stability. Most of the glutamate oxidases studied so far are stable within a temperature range from 30 to 50–55°C [8, 10–12]. Only the glutamate oxidase of *Streptomyces* sp. X-119-6 was stable at 65°C and lost only 50% of its activity at 85°C; however, the enzyme was completely inactivated at 90°C [7].

We incubated the glutamate oxidase of *Streptomyces* sp. Z-11-6 for 20 min at temperatures from 30 to 90°C at pH 5.5, 6.5, or 9.5. Then, the solution was rapidly cooled and glutamate oxidase activity was measured. At pH 5.5, the enzyme was stable at 30–60°C and lost 45% of its activity at 70°C; at pH 6.5, the enzyme was stable at 30–50°C and lost 45% of its activity at 60°C; at pH 9.5, the stability was observed at 30–40°C, and loss of 45% of its activity occurred at 50°C (Fig. 2d). Upon incubation of the enzyme at 90°C, the residual activity was about 5% regardless of the initial pH of the solution.

The effect of metal ions, inhibitors, and chelating agents on the enzyme activity. To study the effect of various agents on glutamate oxidase activity, we added them to the reaction mixture at a concentration of 1 mM and determined the enzyme activity after a 5-min incubation. As seen from Table 1, the enzyme was rather tolerant to metal ions and SH-reagents. *p*-Chloromercuribenzoate (PCMB) caused 18% inhibition, KSCN caused 10% inhibition, and copper chloride and diethyldithiocarbamate caused no inhibition at all (the activity

of previously studied glutamate oxidases is inhibited by PCMB by 45% [2] and by copper chloride by 50% [8].

Enzyme stability during storage. The partially purified enzyme was lyophilized. During storage in the lyophilized state under an argon atmosphere at room temperature for 6 months, the enzyme retained 90% of its initial activity.

In conclusion, the enzyme studied in this work exhibits high heat and pH stability, resistance to copper ions, PCMB, and chelating agents, high substrate specificity and affinity to L-glutamate, and also stability during storage. These properties make the extracellular L-glutamate oxidase of *Streptomyces* sp. Z-11-6 a promising reagent to aid in the development of biosensors for various clinical and biochemical analyses.

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