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Purification and characterization of extracellular glutaminase from *Aspergillus oryzae* NRRL 32567

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ABSTRACT

The glutaminase produced from *Aspergillus oryzae* NRRL 32567 was purified (10.2 folds) using ammonium sulfate fractionation followed by gel filtration on Sephadex G-75. SDS-PAGE of the purified glutaminase showed the presence of one band with a molecular weight of 68 kDa. Optimum pH was 7.0 while a temperature range 30–40 °C was optimal the activity. The highest pH stability was obtained at pH 7.0 while a temperature range 30–40 °C resulted in the highest temperature stability. The apparent K_m value was calculated from the Linenweaver-Burk plot and was found to be 4.5 mM. Glutamine was the preferred substrate for the enzyme and the maximum relative activity of 130% was observed at 2.5% glutamine . Potassium showed a slight increase in activity of glutaminase especially at the concentration of 0.5 mM. While, frerric ions followed by ferrous showed the highest inhibition effect on glutaminase.

1. Introduction

Glutaminase (EC 3.5.1.2) is an amidohydrolase enzyme which generates L-glutamic acid and ammonia from L-glutamine. A variety of microorganisms, including bacteria, yeasts and molds have been reported to produce L-glutaminase (Kashyap et al., 2002; Weingand-Ziadé et al., 2003; Iyer and Singhal, 2008) of which the most potent producers are molds (Balagurunathan et al., 2010). On an industrial scale, glutaminases are mainly produced by the genera Aspergillus and Trichoderma (Yamamoto and Hirooka, 1974; El-Sayed, 2009). *Aspergillus oryzae* is a filamentous fungus, which has an ability to secrete large amounts of hydrolytic enzymes. It is widely used in the manufacture of traditional fermented soy sauce in Asia (Wood, 1977). Moreover, *A. oryzae* is genomically well characterized and considered to be a safe organism for producing of food enzymes because it lacks expressed sequence tags for the genes responsible for aflatoxin production (Fabio et al., 2012). Also, it is widely recognized as preferable L-glutaminase sources because they generally produce extracellular enzymes, which facilitate the enzyme recovery from the fermentation broth (Koibuchi et al., 2000).

In recent years, L-glutaminase has attracted much attention in both pharmaceutical and food industrial applications. In food industry, L-glutaminase is used for the production of glutamic acid, which is the most important amino acid in food manufacture for a delicious taste (O`Mahony and Ishi, 1987; Fukushima and Motai, 1990). The pleasant and palatable tastes of oriental fermented food like soy sauce, miso and sufu are considered to be related to their content of L-glutamic acid accumulated due to the hydrolysis of a protein catalysed by proteolytic enzymes, including L-glutaminase, protease and peptidases (Lu et al., 1996). Salt tolerant L-glutaminases are most valuable in the industrial processes that require high salt environments like the soy sauce fermentation. L-glutaminases from conventional sources (*Aspergillus oryzae*) are markedly inhibited by high salt concentrations as demonstrated by Kumar et al. (2012). Salt tolerant L-glutaminases were patented for use in industrial processes (Sabu et al., 2000). Tumor growth regulation can be achieved by inhibition of both protein and nucleic acid biosynthesis in the cancerous cells due to the lack of availability of any component of these macromolecules. Inhibition of the tumor cell uptake of glutamine is one of the possible ways to stop the growth and this is the best accomplished by the use of L-glutaminase, which breaks down L-glutamine. This in fact, results in a selective starvation of the tumor cells because unlike normal cells they lack properly functioning glutamine biosynthetic machinery (Tanaka et al., 1988). Another important application of L-glutaminase is in biosensors for monitoring the glutamine levels in mammalian and hybridoma cells (Kashyap et al., 2002; Klein et al.,

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2002). The objective of this work was focused on the purification and characterization of glutaminase from *Aspergillus oryzae* NRRL 32567.

2. Materials and methods

2.1. Enzyme

The crude L-glutaminase was prepared from *Aspergillus oryzae* NRRL 32567 (as a new source for glutaminase) as early described by Alian et al. (2015) as following: Fifty milliliters of a medium containing ($g\,L^{-1}$): yeast extract, 20; lactose, 25; glutamine, 5; KH_2PO_4, 1.5; K_2HPO_4, 3; NaCl, 5 and Mg $SO_4 \cdot 7H_2O$, 5 at pH 7.0 were placed in 250 ml Erlenmeyer flasks and autoclaved at 121 °C for 15 min. Each of the sterilized flasks was inoculated with 1 ml spores suspension (1.5×10^7 spores ml^{-1}), placed in a rotary shaker (100 rpm) and the growth was aerobically carried out at 30 °C for 3 days. At the end of the incubation period, the mycelia were recovered from each flask by filtration on Whatman no. 1 (Whatman Ltd., Maidstone, England) and culture filtrate was used as the source of crude glutaminase.

2.2. Assay of L-glutaminase

L-glutaminase activity was determined using the method of Imada et al. (1973) and as modified by Alian et al. (2015) utilizing L-glutamine as substrate and the released ammonia was measured using Nesseler's reagent. An aliquot of 0.5 ml of the sample was mixed with 0.5 ml of 0.04 M L-glutamine solution in the presence of 0.5 ml of distilled water and 0.5 ml phosphate buffer (0.1 M, pH 7.0). Then the mixture was incubated at 37 °C for 30 min and the reaction was stopped by the addition of 0.5 ml of 1.5 M Trichloroacetic acid. To 0.1 ml of the mixture, 3.7 ml of distilled water and 0.2 ml of Nessler's reagent were added. The absorbance was measured at 430 nm using a spectrophotometer, Model 6300 (Jan way LTD., Essex, U.K). A standard graph was plotted using ammonium chloride. One international unit of L-glutaminase was defined as the amount of enzyme that liberates one µmol of ammonia under optimum conditions. One international unit of L-glutaminase was defined as the amount of enzyme that liberates one µmol of ammonia under optimum conditions.

2.3. Determination of enzyme protein

Enzyme protein was spectrophotometrically determined according to the method of Lowry et al. (1951) using Bovine Serum Albumin as a standard.

2.4. Glutaminase purification

2.4.1. Fractionation by ammonium sulfate

Different saturation levels of ammonium sulfate were used (20%, 30%, 40%, 50%, 60%, 70%, 80% and 90%). Known volumes of crude enzyme filtrate were successively treated with such levels of ammonium sulfate for overnight at 5 °C and then centrifuged at 8000 rpm for 20 min (Abdallah et al., 2013). The obtained precipitate was overnight dialyzed in a cellulose bag (Fisher Scientific Company. USA) against sodium phosphate buffer (0.1 M, pH 7.0) at 5 °C under mild agitation. The buffer was changed each 4 h. An aliquot was then collected and analyzed for glutaminase activity and protein content.

2.4.2. Gel filtration chromatography

The dialyzed enzyme solution (1 ml) was added to a Sephadex G-75 column (1.0×45 cm) previously equilibrated with sodium phosphate buffer (pH 7.0, 0.1 M) and eluted with the same buffer without change in ionic strength at a flow rate of 0.6 ml min^{-1}. Fractions of 3.0 ml were collected and both protein content and glutaminase activity were off-line determined. Protein content in the elutent was spectrophotometrically measured at 280 nm (Ali et al., 2009).

2.4.3. Gel electrophoresis (SDS-PAGE)

The purity of L- glutaminase protein was tested by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) using coomassie brilliant blue dye according to the procedure of Ali et al. (2009).

2.5. Characteristics of the purified glutaminase

2.5.1. Optimum reaction temperature

The effect of temperature on purified glutaminase activity was studied by carrying out the enzyme reaction for 30 min at different temperatures (25, 30, 40, 50, 60 and 70 °C) using the same enzyme assay conditions previously described.

2.5.2. Thermal stability

The purified enzyme was incubated at different temperatures (30, 40, 50, 60 and 70 °C) for various lengths of time up to 2.0 h. After cooling, the residual activities were determined under the optimized assay conditions.

2.5.3. Optimum reaction pH

The effect of pH on purified enzyme was determined by carrying out enzyme reaction at different pH levels using different buffer systems (0.1 M): sodium acetate buffer (pH 3.0–5.0), sodium phosphate buffer (pH 6.0–8.0) and boric acid borate (pH 9.0–10) using the same enzyme assay conditions previously described.

2.5.4. pH stability

The purified enzyme was incubated at various pH levels (3.0–10). Such levels were achieved by the application of different buffer systems (0.1 M): sodium acetate buffer (pH 3.0–5.0), sodium phosphate buffer (pH 6.0–8.0) and boric acid borate (pH 9.0–10). Samples were withdrawn at time intervals and the enzyme activity was assayed under the optimized assay condition.

2.5.5. K_m determination

The Michaelis constant (K_m) of the purified glutaminase was determined by measuring the reaction velocities (µM ammonia min^{-1}) at various concentrations of glutamine (mM ml^{-1}) at 30 °C for 30 min. the data were plotted according to Lineweaver and Burk (1934).

2.5.6. Substrate specificity

Different substrates i.e., L-glutamine, D- glutamine, L- asparagine, D- asparagine, L- glutamate (monosodium glutamate) and L-glutamic acid were used. Each one was separately added to the reaction mixture in amount of 0.04 M and glutaminase activity was measured in normal conditions and the relative activity was calculated.

2.5.7. Salt-tolerant

The activity of purified enzyme was assayed in the standard reaction mixture supplemented with NaCl at various concentrations (0–20%, w/v) and the relative activity was calculated.

2.5.8. Effect of various metal ions

Metal ions such as: K^+, Na^+, Cu^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, Ca^{2+}, Hg^{2+} and Zn^{2+} were separately tested for their effect on glutaminase activity. Salts of the different metals were supplemented