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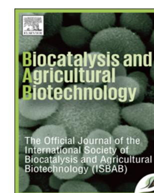

Original Research Paper

Purification and partial characterization of a low molecular weight L-asparaginase produced from corn cob waste

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ABSTRACT

L-asparaginase (E.C.3.5.1.1) is an important enzyme often used to treat acute lymphoblastic leukemia. This paper describes the production, purification and partial characterization of L-asparaginase by *Streptobacillus* sp. KK2S4. The highest L-asparaginase production was achieved with 0.2% (w/v) of lactose, 0.1% (w/v) of sodium nitrate, 6% (w/v) of pre-treated corn cob powder and 4% (v/v) of KK2S4 bacterial suspension in 50 ml of MM9 media at pH 5 and 40 °C. The enzyme was purified by ammonium sulfate precipitation followed by DEAE-cellulose and Sephadex G-50 column chromatography. The specific activity of the pure enzyme was recorded to be 21.77 U/mg with 39.58-fold purification and 39% of yield. SDS-PAGE demonstrated a single band with molecular weight of 11.2 kDa. The optimum activity of purified enzyme was recorded at pH 8.5 and 35 °C. This is the first report of a very low molecular weight microbial L-asparaginase produced from corn cob as the main carbon source.

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1. Introduction

L-asparaginase (EC 3.5.1.1) is a universal and essential component utilized in the chemotherapy regimens for the treatment of the cancerous lymphoid system, non-Hodgkin's lymphoma and childhood acute lymphoblastic leukemia (ALL) (Kumar et al., 2011; Muller and Boos, 1998) and to reduce the acrylamide formation in the food processing industry (Mario et al., 2007; Becalski et al., 2003; Friedman, 2003; Zyzak et al., 2003; Lingnert et al., 2002). The market names for L-asparaginase are ONCASPAR, KIDROLASE, ERWINASE and ELSPAR. According to the biochemical properties and the sequence homology, L-asparaginase can be produced from three families of organisms including bacteria, plant and also *Rhizobium etli*. L-asparagine is an important growth factor for the malignant cells. The capability of the L-asparaginase to degrade the L-asparagine into L-aspartic acid and ammonia (Rani et al., 2012; Verma et al., 2007; Savitri and Azmi, 2003; Borek and Jaskólski, 2001) gives the anti-leukemic characteristic to this enzyme as the tumor cells are unable to synthesize asparagine from the aspartic acid (Kamble et al., 2012). Other than that, L-asparaginase is one of the most widely studied therapeutic enzymes around the globe for the treatment of ALL (Borah et al., 2012). The utilization of L-asparaginase from different organisms can cause allergic response during chemotherapy (Kumar et al., 2011; Wink et al., 2008). Therefore, the purpose of this research was to obtain low cost L-asparaginase (from waste) with similar therapeutic effect. In this research, L-asparaginase was produced from corn cob cellulosic waste through microbial fermentation. It is highly desirable especially when it can be produced from the waste materials which on one hand allows recycling of the waste to yield valuable material and on the other hand maintains green environment. Further, the research aimed to proceed through a series of downstream processes to purify the enzyme and partially characterize it with special emphasis on its molecular weight and some parameters related to its stability.

2. Materials and methods

2.1. Corn cob pretreatment and inoculum preparation

The corn cob was collected and sun dried for two weeks to make sure it was fully dry. The dried corn cob was powdered using a grinding machine (Xingang, F220) and subsequently pre-treated with 2 N NaOH by soaking overnight. The substrate was filtered through three layers of muslin cloth and washed several times with tap water until the pH was stable at around 7.0. The pH was further adjusted by using HCl and NaOH. The color of the substrate turned light yellow after washing and filtration. Bacterial suspension (*Bacillus sp.* KK2S4, previously isolated from soil) was prepared for inoculation in this study according to Makky et al. (2013). About 50 ml of nutrient broth media was prepared into a

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100 ml conical flask and autoclaved for 20 min at 121 °C, brought to room temperature and pure KK2S4 isolate was inoculated and incubated at 37 °C for 48 h (Makky et al., 2013).

2.2. Media preparation

Around 50 ml of MM9 (Gibco® M9 Minimal Salts (2X) media Cat. No. A13744-01) was transferred into two 100 ml Erlenmeyer flasks containing 1.5 g of treated corn cob powder and was autoclaved at 121 °C for 20 min. Then, 5 ml of bacterial suspension was inoculated into the Erlenmeyer flasks. For the control production media, no microbial isolate was added into the production media. The production media was incubated at 37 °C for 48 h.

2.3. Cell-free crude enzyme preparation

After incubation for 48 h, the production media was transferred into a 50 ml of conical centrifuge tube and centrifuged at 5000*g* for 6 min at 4 °C to obtain cell-free filtrate (CFF) which was used as crude enzyme (Makky et al., 2013; Jain et al., 2012).

2.4. Assay of L-asparaginase

L-asparaginase assay was performed by following Nessler's reaction as described by Imada et al. (1973). This method was used to determine the amount of ammonia liberated from L-asparagine in the enzyme-catalyzed reaction. The reaction was initiated by adding 0.5 ml of supernatant, 0.5 ml of 0.04 M L-asparagine, 0.5 ml of 0.5 M acetate buffer, pH 5.4 and 0.5 ml of distilled water in a test tube and incubated at 37 °C for 30 min. About 0.5 ml of 1.5 M trichloroacetic acid (TCA) was added into the sample to stop the reaction. The zero time control was prepared by adding TCA before incubation. The reaction mixture was centrifuged at 5000*g* for 5 min and 0.1 ml of the supernatant was added to the tube containing 0.2 ml Nessler's reagent and 3.7 ml of distilled water and incubated at room temperature for 20 min. The optical density was measured at 450 nm using a microplate reader (Infinite M200 Pro TECAN, Switzerland) to determine the ammonia released in the supernatant.

2.5. Protein determination

Protein was determined either by measuring OD for monitoring the column elute at 280 nm or by the method of Lowry et al. (1951) using Folin reagent for enzyme specific activity calculation.

2.6. Optimization of enzyme productivity

To study the effect of substrate concentration on enzyme productivity, different amounts of treated corn cob substrate (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 g) were weighed and added into six Erlenmeyer flasks, respectively containing 50 ml of MM9 media. About 5 ml of most potent isolate KK2S4 was transferred into each flask and cultured under standard temperature and pH. While for the study of the effect of inoculum size on the L-asparaginase productivity, 2, 4, 6, 8 and 10 ml of KK2S4 suspension were prepared. All inocula were pipetted into 50 ml of MM9 media containing 1.5 g of treated substrate. To determine the optimum temperature, the inoculates containing 50 ml of MM9 media, 1.5 g of treated substrate and 5 ml of KK2S4 suspension were cultured under 7, 20, 30, 37, 40, and 50 °C (Sunitha et al., 2010).

2.7. Enzyme production

The production medium was prepared by adding 1.5 g of sodium nitrate, 15 ml of 20% lactose and 90 g of treated corn cob substrate into 1.5 L of MM9 media. The pH of the medium was adjusted to 5.0 by using HCl or NaOH. After autoclave, 60 ml of bacterial isolate KK2S4 was transferred into the medium inside a bioreactor tank and incubated at 40 °C for 48 h to complete the fermentation (Makky et al., 2013).

2.8. Purification of crude enzyme

Modified method of Borah et al. (2012) was used for the purification of crude enzyme. The production media was centrifuged at 5000*g* at 4 °C for 6 min to obtain the supernatant. The protein was precipitated by adding solid ammonium sulfate with slow and continuous stirring by a magnetic stirrer until it reached to 70% saturation and the mixture was left at 4 °C overnight for complete precipitation. The chilled contents were transferred into a 50 ml of falcon tube and centrifuged at 12,000*g* at 4 °C for 20 min. The supernatant was discarded and the pellet was redissolved in minimum volume of 0.5 M acetate buffer, pH 5.4, transferred into a dialysis tube and left for 3 h fully covered with sucrose powder at 4 °C.

2.9. DEAE-cellulose column chromatography

The dialyzed sample was loaded onto a diethylaminoehtyl (DEAE) cellulose column (2.5×30 cm^2; 200 ml) pre-equilibrated with 0.5 M acetate buffer followed by washing with the same buffer. The active fractions were pooled together, assayed, concentrated with 100% ammonium sulfate, dialyzed and subjected to second round of column chromatography.

2.10. Sephadex G-50 column chromatography

The purified active fraction obtained from the step above was loaded onto a Sephadex G-50 column (3×20 cm^2; 150 ml) pre-equilibrated with two column volumes of 0.5 M acetate buffer, pH 5.4. Eventually, the fractions were eluted with the same buffer and assayed for the protein content and enzyme activity at 700 and 450 nm, respectively.

2.11. Purity and molecular weight determination by SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the purified enzyme was performed to determine its molecular weight following the method of Laemmli (1970). The electrophoresis was performed on 10% of separating gel (pH 8.8) and 4% of stacking gel (pH 6.8) and 1X Tris-base (pH 8.3) as running buffer containing 0.1% of SDS and at 200 V and 60 mA of current for 45 min. The gel was stained with Coomassie Brilliant Blue R-250 (Bio-Rad, France). Promega Broad Range Protein Molecular Weight Markers (V849A) consisting of 9 precisely sized recombinant proteins of molecular weights 225, 150, 100, 75, 50, 35, 25, 15 and 10 kDa with protein concentrations of 0.1 μg/μl were used to compare the molecular weight of the purified enzyme.

2.12. Effect of temperature

The purified enzyme was assayed under varying temperature, maintaining other standard parameters. The incubation temperatures were set to 7, 30, 40, 50 and 80 °C for different reaction mixtures and incubated for 20 min (Borah et al., 2012). The reaction was terminated by adding TCA and the absorbance was measured at 450 nm by using a Genesys 10S UV–vis spectrophotometer.

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