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Production, Isolation, and Purification of L-Asparaginase from *Pseudomonas Aeruginosa* 50071 Using Solid-state Fermentation

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The L-asparaginase (E. C. 3. 5. 1. 1) enzyme was purified to homogeneity from *Pseudomonas aeruginosa* 50071 cells that were grown on solid-state fermentation. Different purification steps (including ammonium sulfate fractionation followed by separation on Sephadex G-100 gel filtration and CM-Sephadex C50) were applied to the crude culture filtrate to obtain a pure enzyme preparation. The enzyme was purified 106-fold and showed a final specific activity of 1900 IU/mg with a 43% yield. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the purified enzyme revealed it was one peptide chain with M_r of 160 kDa. A Lineweaver-Burk analysis showed a K_m value of 0.147 mM and V_{max} of 35.7 IU. The enzyme showed maximum activity at pH 9 when incubated at 37ºC for 30 min. The amino acid composition of the purified enzyme was also determined.

Keywords: Amino acid composition, L-asparaginase, Production, *Pseudomonas aeruginosa* 50071, Purification

Introduction

L-Asparaginase (E. C. 3. 5. 1. 1) is present in many animal tissues, bacteria, plants, and in the serum of certain rodents, but not in mankind. L-Asparaginase is produced by a large number of microorganisms that include *E. coli* (Derst *et al*., 1994; Mercado & Arenas, 1999), *Erwinia cartovora* (Maladkar *et al*., 1993; Aghaiypour *et al*., 2001), *Enterobacter aerogenes* (Mukherjee *et al*., 2000), *Corynebacterium glutamicum* (Juan *et al*., 1990), *Candida utilis* (Kil *et al*., 1995), *Staphylococcus aureus* (Muley *et al*., 1998), *Thermus thermophilus* (Prista & Kyridio, 2001), and *Pisum sativum* (Siechiechowcicz & Ireland, 1989). L-Asparaginase catalyzes the hydrolysis of L-asparagine into L-aspartate and ammonia. The precise mechanism of its action is still unknown although hydrolysis proceeds in two steps via a beta-acyl-enzyme intermediate (Fig. 1) (Hill *et al*., 1967). L-Asparaginase is produced throughout the world by submerged fermentation (SF). This technique has many disadvantages, such as the low concentration production, and consequent handling, reduction, and disposal of large volumes of water during the downstream processing. Therefore, the SF technique is a cost intensive, highly problematic, and poorly understood unit operation (Datar, 1986). Solid-state fermentation is a very effective technique as the yield of the product is many times higher when compared to that in SF (Arima, 1964), and it also offers many other advantages (Lonsane *et al*., 1985). Microbial asparaginases have been particularly studied for their applications as therapeutic agents in the treatment of certain types of human cancer (Gallogher *et al*., 1989). L-Asparaginase from two bacterial sources (*E. coli* and *Erwinia carotovora*) is currently in clinical use for the treatment of acute lymphoblastic leukemia (Keating *et al*., 1993). It is also used for the treatment of pancreatic carcinoma (Yunis *et al*., 1977) and bovine lymphomosarcoma (Mosterson *et al*., 1988). A partially-purified L-asparaginase from *Aspergillus terreus* possesses antitumor property against Ehrlich's ascites

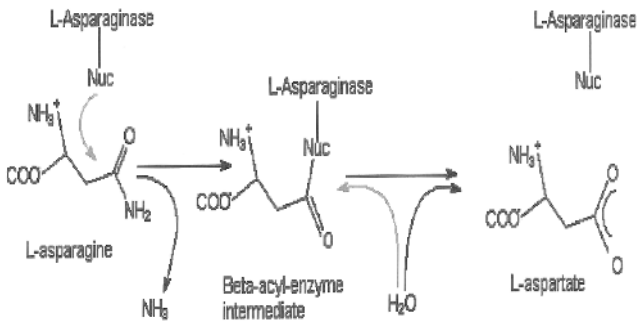

Fig. 1. Schematic illustration of the reaction mechanism of L-asparaginase. (Hill *et al*., 1967)

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in susceptible Swiss mice. From an observation of the toxicity and immunological responses, the enzyme may be non-toxic and have myelosuppressive/immunosuppressive (Ali *et al*., 1994). Therefore, the aim of the present work is the discovery of a new L-asparaginase producer that is serologically different from the previously reported ones, but one that has similar therapeutic effects.

Materials and Methods

Materials L-asparagine was kindly provided by Sigma (St. Louis, USA). Sephadex G-100, CM-Sephadex C50, and standard proteins for SDS-gel electrophoresis were purchased from Pharmacia, Uppsala, Sweden. All of the other chemicals were of analytical grade.

Microorganism media and growth conditions The Bacterial strain of *Pseudomonas aeruginosa* 50071 was kindly provided by the Microbiological Resource Center (MIRCEN), Ain Shams University, Cairo, Egypt. The stock culture was maintained on tryptic soy agar slants that contained tryptic casein Bios D (1.5%), Soy peptone (0.5%), NaCl (0.5%), and agar Bios U (1.5%). The culture was incubated at 37ºC for 18 h and stored at 4ºC. Transfers from the single slant cultures at 48 h old were taken into 50 ml aliquots of the defined inducible medium. These were placed in 250 ml Erlenmeyer flasks and used as the standard inocula (3 ml/flask), unless otherwise indicated to initiate growth and give an initial OD at A_{540} of 0.08.

Cultivation was achieved by solid-state fermentation (SSF) as previously reported by Ramesh and Lonsane (1987). The medium that was used for the cultivation of *Pseudomonas aeruginosa* 50071 under (SSF) had the following composition: 10 g Soya bean meal of 0.4-0.8 cm particle size were moistened with 10 ml of 0.01 M phosphate buffer pH 7.4, and placed in 250 ml Erlenmeyer flasks. The fermentation media were sterilized by autoclaving for 15 min at a pressure of 15 Ib/inch to raise the temperature to 121ºC. The flasks were inoculated with 3 ml of the prepared bacterial suspension and incubated under static conditions at 37ºC for 4 d. The extracellular crude enzyme was prepared at the end of the fermentation period by the addition of 90 ml of a 0.01 M phosphate buffer pH 7 to the fermented medium, shaking for 15 min followed by centrifugation at 8,000 rpm for 20 min. The cell free supernatant was used as the crude enzyme preparation.

Purification of L-asparaginase The purification was carried out at 4ºC on the crude extract, according to the modified method of Distasio *et al*. (1976).

Ammonium sulfate fractionation Finely powdered ammonium sulfate was added to a 80% saturation. The mixture was left for 12 h at 4ºC, followed by centrifugation at 8,000 rpm for 20 min at 4ºC. The precipitate was dissolved in a 0.01 M phosphate buffer pH 8.5 and dialyzed overnight against the same buffer at 4ºC.

Sephadex G-100 gel filtration The dialyzed ammonium sulfate fraction was applied to a Sephadex G-100 column that was pre-equilibrated with a 0.01 M phosphate buffer pH 8.5. The protein elution was done with the same buffer at a flow rate of 5 ml/min. The fractions were collected using a fraction collector (LKB Ultorace) at 4ºC. It was assayed for protein at 280 nm as well as for enzyme activity. The active fractions were pooled, dialyzed against the 0.01 M phosphate buffer pH 8.5, and concentrated.

CM-Sephadex C50 ion-exchange chromatography The concentrated enzyme solution was applied to the column of CM-Sephadex C50 that was pre-equilibrated with a 0.01 M phosphate buffer, pH 8.5. It was eluted with the NaCl gradient (0.1-0.5 M) and 0.1 M borate buffer, pH 7. The active fractions were collected, dialyzed, concentrated, and lyophilized. This preparation was used in the subsequent step.

The enzyme was assayed by the direct nesslerization method according to the method of Sinha *et al*., (1991). One L-asparaginase unit (IU) is defined as the enzyme amount, which liberates 1 µmol of the ammonia/min under optimal assay conditions. The protein concentration was determined by the modified Lowry's method (Kim *et al*., 2002).

Disc-PAGE A slab gel electrophoresis was carried out using a 15% polyacrylamide gel (pH 7.8) with a 5% top gel (pH 6.2). After electrophoresis in a Tris-glycine buffer (pH 8.3) at 200 V for 7 h at 70ºC, the proteins in the gel were stained with Coomassie brilliant blue R-250 and destained (El-Gamal *et al*., 2001).

Molecular weight determination Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a 3-mm slab gel of 6% acrylamide in a Tris-borate buffer pH 7.1 containing 0.1% SDS. The gels were stained with 0.025 Coomassie brilliant blue R-250 and destained (Stegemann, 1979). The following standard proteins were used for the molecular weight determination under identical conditions: lysozyme (14 kDa), carbonic anhydrase (31 kDa), ovalbumin (42 kDa), bovine serum albumin (67 kDa), and phosphorylase (97 kDa).

Determination of amino acid composition This was carried out on the purified enzyme using a Beckman Amino Acid Analyzer (Model 119 GL), according to the method of Speckman *et al*., (1958).

Results

The partial purification of the L-asparaginase crude extract that was affected by the ammonium sulfate (80%) precipitation showed that most of the enzyme activity was preserved in the precipitate. The total protein decreased from 4,150 to 674 mg in the ammonium sulfate precipitation step. The specific activity increased to 497 and 1,900 IU/mg after the Sephadex G-100 and Sephadex C50 steps, respectively (Table 1).

Figure 2 shows the profile of the ammonium sulfate fraction purification on Sephadex G-100 gel filtration column chromatography. Although this fraction contained different protein molecules, only one peak showed activity for L-asparaginase. Also, the purification of the enzyme rich

Table 1. Purification profile of L-asparaginase from Pseudomonas aeruginosa 50071

Step	Collected volume (ml)	Total activity (IU)	Total Protein (mg)	Specific activity (IU/mg)	Purification (fold)	Yield (%)
Crude extract	450	74300	4150	17.9	0.00	100
$(NH_4)_2SO_2$ precipitation	275	63100	674	93.7	5.2	85
Gel filtration on Sephadex G100 column	175	45200	91	497	27.7	60.8
CM-Sephadex C50 column	100	32300	17	1900	106	43

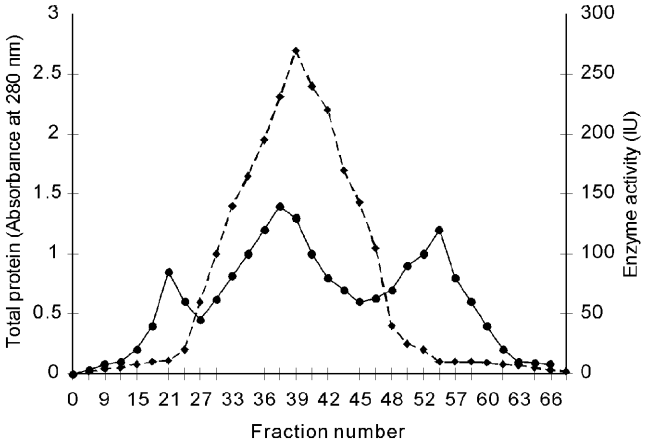

Fig. 2. First gel filtration chromatography of L-asparagiase. The dialyzed ammonium sulfate precipitate was chromatogrophed on Sephadex G-100. Total protein was monitored at 280 nm. The fractions were assayed for the enzyme activity. (Dashed line, enzyme activity).

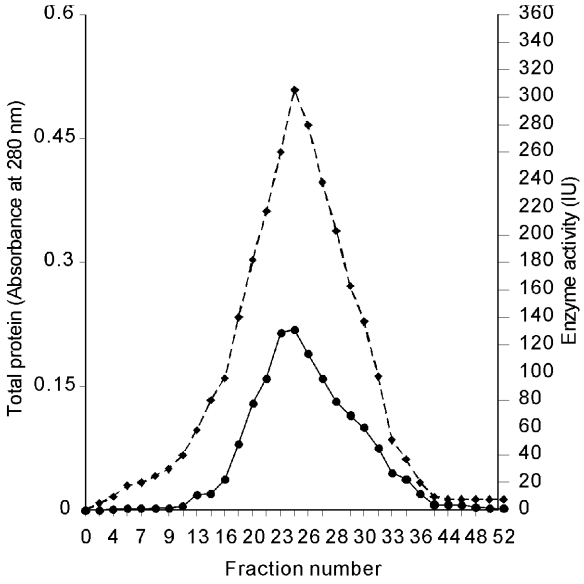

Fig. 3. Second CM-Sephadex C50 chromatography of L-asparaginase. The first gel filtration G-100 collected fraction was applied to CM-Sephadex C50. Total protein was monitored at 280 nm and the fractions was assayed for L-asparaginase activity. (Dashed line, enzyme activity).

fractions of the Sephadex G-100 gel filtration on the CM-Sephadex C50 column is shown in Fig. 3. A sharp distinctive peak of L-asparaginase activity, which fits with only one protein peak, was obtained.

DISC-PAG of the enzyme preparation from different purification steps showed that the resolved electrophoretic bands were progressively improved from the crude extract to the final step of the CM-Sephadex C50 column. It revealed only one distinctive band that was indicated by the pure preparation of L-asparaginase (Fig. 4).

Molecular weight of L-asparaginase SDS-PAGE showed that the enzyme is one band with electrophoretic mobility of 0.48. By using different standard proteins with known molecular weights, it was discovered that the apparent molecular weight of *Pseudomonas aeruginosa* 50071 L-asparaginase was 160 kDa (Fig. 5).

Kinetic properties of the purified L-asparaginase The pH influence on the L-asparaginase activity was studied using a 0.01 M phosphate buffer of different pH values, ranging from 2.0 to 11.The enzyme activity gradually increased until pH 9, at which time the maximum activity was observed. At higher pH, the enzyme activity decreased (Fig. 6). The reaction rate of L-asparaginase was measured at various temperatures. Maximum activity was obtained at 37°C. At higher temperatures, the reaction rate declined sharply.

The effect of the incubation time on L-asparaginase activity was studied in the ranges of zero to 50 min (Figs. 7 and 8). L-Asparaginase activity increased as the incubation time increased. The activity ran at maximum for 30 min and decreased as the time increased. A Lineweaver-Burk analysis gave K_m of 0.147 mM and a V_{max} value of 35.7 IU (Fig. 9).

Amino acid composition Table 2 shows the amino acid contents of the purified *Pseudomonas aeruginosa* 50071 L-asparaginase. The purified enzyme was rich in glycine and glutamic acid.

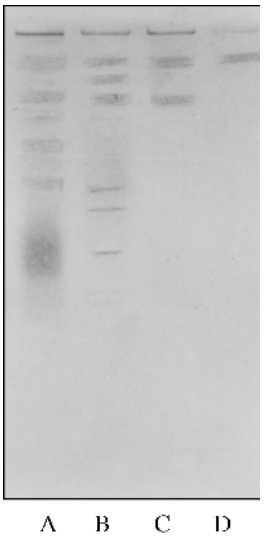

Fig. 4. Polyacrylamide gel electrophoresis of L-asparaginase from *Pseudomonas aeruginosa* 50071. Electrophresis of the enzyme was carried out on a 15% polyacrylamide gel in the absence of SDS and the gel was stained with Coomassei blue R-250. Lane A, crude extract; Lane B, ammonium sulfate fraction; Lane C, Sephadex G-100 gel filtration fraction; Lane D, CM-Sephadex C50 fraction.

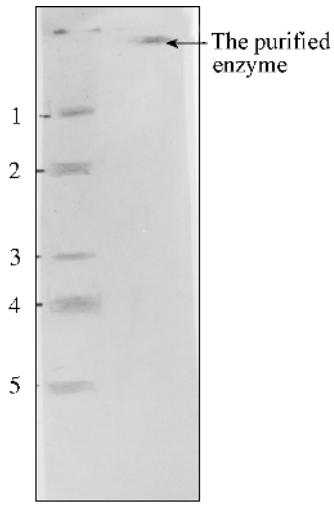

Fig. 5. PAGE-SDS of L-asparaginase from *Pseudomonas aeruginosa* 50071. Electrophresis was carried out on a 6% polyacrylamide containing 0.1% SDS. The gel was stained with Coomassie blue R-250. Lane A included the following standard proteins: 1. phosphorylase (M_r 97 kDa), 2. bovine serum albumin (M_r 67 kDa), 3. ovalbumin (M_r 42 kDa), 4. carbonic anhydrase (M_r 31 kDa), 5. lysozyme (M_r 14 KDa). Lane B contained CM-Sephadex C50 column purified enzyme.

Discussion

Purification of *Pseudomonas aeruginosa* 50071 L-asparaginase was achieved by using 80% ammonium sulfate saturation, Sephadex G-100 gel filtration, and CM-Sephadex C50 cation exchange column, respectively. The specific activity increased from 17.9 to 1900 IU/mg for the crude extract and the final preparation, respectively. The final preparation was examined using DISC-PAGE and SDS-PAGE, which revealed that it contained one protein band with M_r of 160 kDa. In this respect, the enzyme was more or less higher than that obtained from *Corynebaterium glutamicum* (Mesas *et al.,* 1990) with M_r of 80 kDa. Other bacterial species produced L-asparaginase of more than one component with variable molecular weights (Rozalska, 1989). Maximum L-asparaginase activity occurred when it was incubated with an optimum substrate concentration at pH 9. A similar pH value was obtained for *E. coli* (Castaman & Rodeghiero, 1993 and Liboshi *et al*., 1999), *Pseudomonas aeruginosa* 10145 (Roberts *et al*., 1968), and many other microbial asparaginase activities (Balcao *et al*., 2001). A temperature profile showed

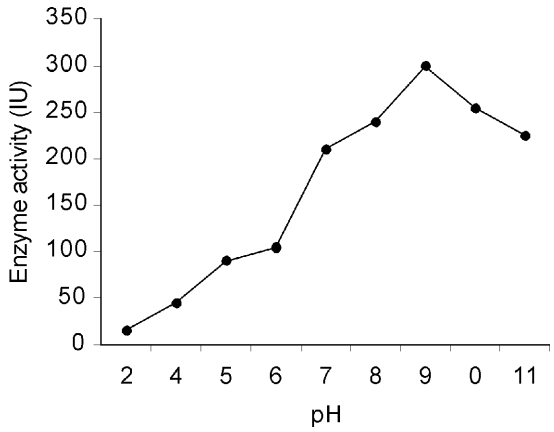

Fig. 6. Effect of pH on L-asparaginase activity. This was studied in the range value from 2-11 using 0.01 M phosphate buffer.

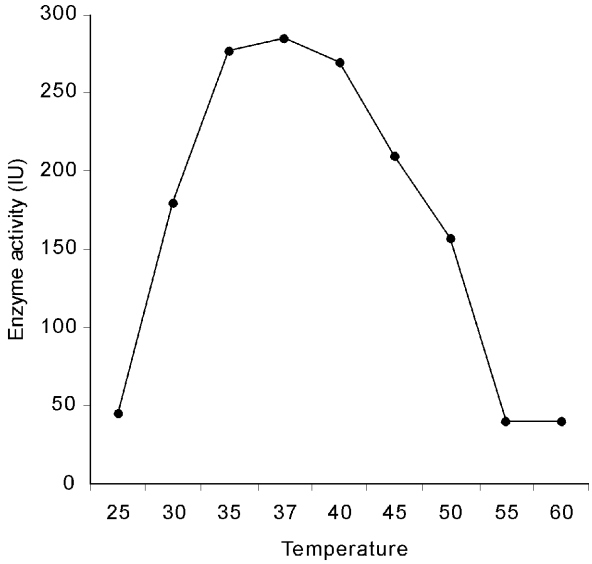

Fig. 7. Effect of temperature on L-asparaginase activity. This was studied in the range of 25-60ºC in a 0.01 M phosphate buffer, pH 9.

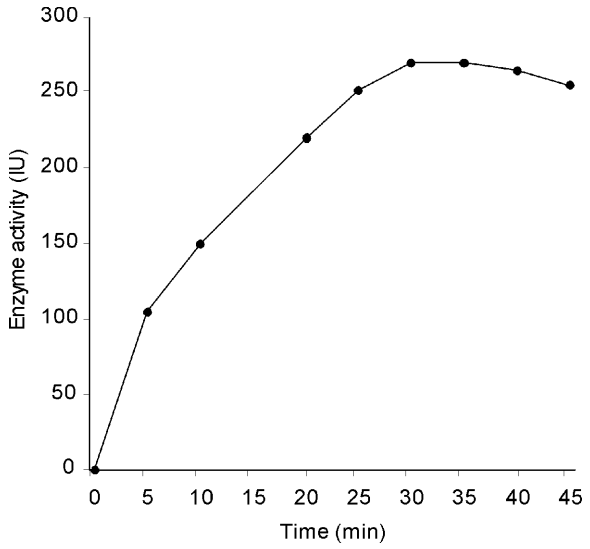

Fig. 8. Effect of incubation time on L-asparaginase activity. This was studied in the range value of 5 to 50 min at 37°C and pH 9.

Table 2. Amino acid contents (mole %) of the purified Pseudomonas aeruginosa 50071 L-asparaginase

Amino acid	mol %
Aspartic acid	8.70
Threonine	2.71
Serine	4.07
Glutamic acid	12.9
Proline	2.71
Glycine	17.1
Alanine	3.9
Cystine	1.64
Valine	2.1
Methionine	6.94
Isoleucine	1.97
Leucine	4.25
Tyrosine	1.72
Phenylalanine	3.07
Histidine	3.99
Lysine	5.07
Arginine	3.32

that the enzyme had maximum activity at 37°C, and more than 52% activity was attained at 50°C. Similar results were recorded for asparaginases from *Pseudomonas stutzeri* MB-405 (Manna *et al*., 1995), *E. carotovora* (Maladkar *et al*., 1993), and *Staphylococcus* (Sobis & Mikucki, 1991). Also, Qian *et al.*, (1996) proved that *E. coli* L-asparaginase lost its activity more rapidly at higher temperatures. On the other hand, L-asparaginase from *chrombacteriaceae* had maximum activity at 20°C (Roberts *et al*., 1972). Incubation of L-asparaginase at 37°C for different times showed that the activity reached its maximum at 30 min. Based on the Lineweaver-Burk analysis, the K_m and V_{max} values of L-asparaginase from *Pseudomonas aeruginosa* 50071 were 0.147 mM and 35.7 IU, respectively. This indicates the high affinity of the enzyme to the substrate. L-Asparaginase of different microorganisms has different substrate affinities and probably plays different physiological roles in the enzyme activity. Higher K_m values (2.5 mM and 3.5 mM) for L-asparaginase from *C. glueamicum* and *E. coli*, respectively, have been reported (Willis and Woolfolk, 1974). On the other hand, a lower K_m value (0.074 mM) was obtained for L-asparaginase from *Vibrio succinogenes* (Willis and Woolfolk, 1974).

The quaity of *P. aeruginosa* 50071 L-asparaginase was assessed for its amino acid contents. The purified enzyme was rich in glycine and glutamic acid. Relatively higher amounts of aspartic acid, methionine, lysine, leucine, and serine were present. Qian *et al.* (1996) reported that aspartic acid protects the active site of *E. coli* L-asparaginase. Finally, this work gives promising results on the possible production of L-

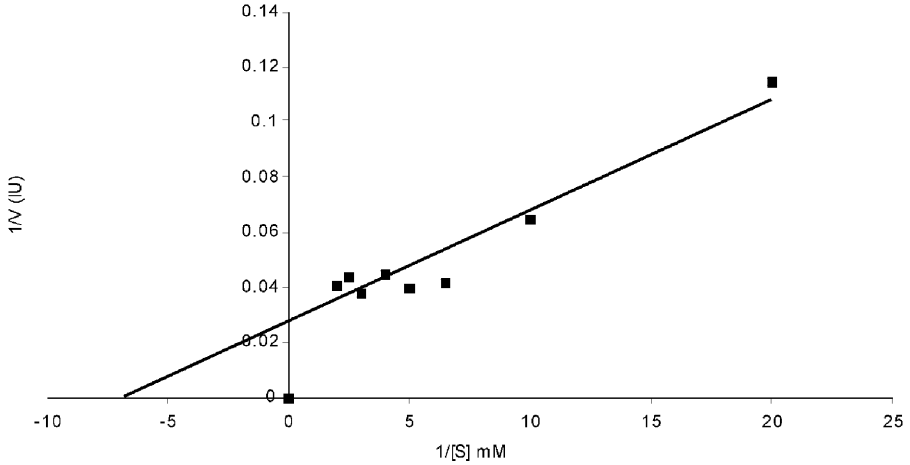

Fig. 9. Determination of K_m and V_{max} of L-asparaginase from *Pseudomonas aeruginosa* 50071. A Lineweaver-Burk plot was used to detect the dependence of L-asparaginase activity on the L-asparagine concentration.

asparaginase from *Pseudomonas aeruginosa* 50071 under solid-state fermentation. This is reported here for the first time. Economically, this enzyme could be produced from cheap, untreated biomass residues. The excellent properties of this enzyme, such as the activity at the alkaline pH range at 37°C, make it extremely valuable in the chemotherapeutic treatment of leukemia.

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