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EXTRACELLULAR GLUTAMINASE-FREE L-ASPARAGINASE FROM *TRICHODERMA VIRIDE* F2: PURIFICATION, BIOCHEMICAL CHARACTERIZATION AND EVALUATION OF ITS POTENTIAL IN MITIGATING ACRYLAMIDE FORMATION IN STARCHY FRIED FOOD

*Ali M. Elshafei and Dina H. El-Ghonemy**

Address(es):
Microbial Chemistry Department, Genetic Engineering and Biotechnology Research Division, National Research Centre, 33 El Buhouth St., Giza 12622, Egypt.

*Corresponding author: delghonamy@yahoo.com

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ABSTRACT

L-asparaginase is an antitumor agent that suppresses cancer cell growth by eliminating L-asparagine from malignant cells. However, the intrinsic glutaminase activity is responsible for significant life-threatening adverse effects. Therefore, glutaminase-free L-asparaginase is far required to improve the therapeutic efficacy of L-asparaginase treatment. L-asparaginase was also used to combat the development of acrylamide in foods rich in carbohydrates cooked at high temperatures. Therefore, this study explores the purification and characterization of glutaminase-free L-asparaginase from *Trichoderma viride* F2 using agro-industrial residues as substrate. The enzyme was purified 36-folds with 688.1 U/mg specific activity and a final yield of 38.9% through ethanol precipitation, gel filtration on Sephadex G-100 followed by Sephadex G-200. The purified L-asparaginase is monomeric with a molecular mass of 57 kDa and exhibited optimum activity at pH 7.5 and 37 °C, which is relatively close to the human body's internal environment. The purified L-asparaginase showed high affinity and catalytic efficiency towards its natural substrate L-asparagine with K_m and V_{max} of 1.2 mM and 71.3 U/mL, respectively, and did not exhibit any intrinsic glutaminase activity. Among the salts tested, the univalent cations Na^+ and K^+ enhanced the activity by 145.7% and 163.5%, respectively, while the presence of Ag^+ and Fe^{+3} displayed a considerable loss in activity. The enzyme showed a good anti-oxidant activity with IC_{50} of 66.1µg/mL and was able to convert L-asparagine exist in potatoes to L-aspartic acid and ammonia, suggesting its use as anti-carcinogenic agent and as potential food industry candidate to mitigate acrylamide content in starchy fried food.

Keywords: Glutaminase-free L-asparaginase; *Trichoderma viride* F2; purification; kinetic properties; anti-oxidant activity, acrylamide reduction

INTRODUCTION

L-asparaginase (L-asparagine amidohydrolase, EC.3.5.1.1) is an antitumor agent that is widely used in various metastasis treatment protocols such as that of acute lymphoblastic leukaemia, breast cancer and non-Hodgkin lymphomas **(Benchamin *et al.* 2019)**. The use of L-asparaginase is based on its ability to convert L-asparagine, an amino acid important for lymphoblast growth, into ammonia and L-aspartic acid in serum and cerebrospinal fluid **(Kalyanasundaram *et al.* 2015; Ray *et al.* 2019)**. Since lymphoblasts are unable to synthesize endogenous L-asparagine, because of the absence of asparagine synthetase, they become entirely dependent on external L-asparagine for protein synthesis and survival. As a consequence, cells that cannot express enough asparagine synthetase are destroyed **(Kotzia and Labrou 2007)**. The relevance of this enzyme is also evident from its high global demand of 380 million USD in 2017, which is expected to increase to 420 million USD by 2025 **(Alam *et al.* 2019)**. Apart from its medicinal uses, L-asparaginase has recently gained increased attention as a food processing aid that can actively degrade acrylamide (C_3H_5NO), a human carcinogenic molecule, and hence reducing its formation in starch-based food that are baked, roasted or fried at temperatures above 100 °C **(Alam *et al.* 2019)**. The formation of acrylamide was attributed to the Millard reaction between free L-asparagine and carbonyl compounds at high temperatures **(Xu *et al.* 2016)**. Therefore, the production of acrylamide was prevented by the depletion of L-asparagine by L-asparaginase **(Pedreschi *et al.* 2008)**.

L-asparaginase is widely distributed in nature from prokaryotic to vertebrates **(Moorthy *et al.* 2010)**. It is generally found in microbes **(Abhinav *et al.* 2012; Elshafei *et al.* 2012)** and some important plants **(Mohamed *et al.* 2015; Mohamed *et al.* 2016)**. A few rodents have the potential to produce this enzyme in their blood serum, but humans do not have such an important anti-carcinogenic agent **(Pieters *et al.* 2011)**. L-asparaginase often acts as an intracellular enzyme, however, extracellular L-asparaginases are considered to be more advantageous and favoured over the intracellular L-asparaginase because they can be produced extensively under normal conditions in the culture broth and can be economically purified **(Amena *et al.* 2010)**. L-asparaginases from *Escherichia coli* and *Erwinia chrysanthemi* have been produced on an industrial scale and successfully were used in treating acute lymphoblastic leukaemia (ALL) and non-hodgkin lymphoma (NHL) **(Makky *et al.* 2013; Krishnapura *et al.* 2016)**. Unfortunately, patients rarely encounter a therapeutic response without some evidence of adverse effects such as pancreatitis, hyperglycemia and different hypersensitivity reactions **(Kaminsky 2017)** that might be attributed to the intrinsic L-glutaminase activity of 3-10% **(Chan *et al.* 2014)**. Therefore, the search for glutaminase-free L-asparaginase with efficient antineoplastic activity but with a reduced or no adverse reaction has recently gained much interest of modern researchers **(Narta *et al.* 2007)**. Hence, the current work focuses on the purification and characterization of an extracellular glutaminase-free L-asparaginase from a terrestrial fungus *Trichoderma viride* F2 grown under solid-state fermentation (SSF). The assessment of L-asparaginase antioxidant activity compared to ascorbic acid was evaluated. In addition, the potential for enzymatic acrylamide reduction in potatoes using the purified *T. viride* F2 L-asparaginase was investigated.

MATERIAL AND METHODS

Chemicals

L-asparagine, L-glutamine, D-asparagine, D-glutamine, bovine serum albumin (BSA), molecular mass markers and 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) were bought from Sigma Chemicals Company (St. Louis, USA). Nessler's reagent was purchased from Merck (Germany). Sephadex G-100 and Sephadex G-200 were bought from Pharmacia Biotech (Uppsala, Sweden). Wheat bran (WB) and rice husk (RH) were kindly donated from the local Arma Food Industry and Alfyoum Co., for soap and animal feed, respectively. All other

chemicals and reagents were of the highest analytical quality and were bought from standard chemical companies.

Microorganism

The filamentous fungus *Trichoderma viride* F2, previously reported as a hyper-asparaginase producing terrestrial fungus (**Elshafei and El-Ghonemy 2015**), was periodically subcultured every 3-4 weeks interval at 28 ˚C on potato dextrose agar slants (PDA), and maintained at 5 ˚C.

Aflatoxins detection

The determination of aflatoxins for *Trichoderma viride* F2 was performed using the high-performance liquid chromatography (1100 HPLC; Agilent Technology, USA) as described by **AOAC Method 971.24 (2000)**. Data developed were reported using the Millennium Chromatography Manager Software.

Enzyme production and extraction

Spore suspension was prepared from a seven day-old culture on PDA medium with 5 mL of sterilized saline water containing 0.1% Tween 80. Two mL of the suspension (1×10^8 spores/mL) was transferred to Erlenmeyer flask (500 mL) containing 10 g of RH and WB in the ratio of 3.0:2.0 moistened (up to 75%) with previously optimized Czapek Dox medium (**Elshafei and El-Ghonemy 2015**) composed of (g/L): glucose 10; casein 15; $NaNO_3$ 1.5; L-asparagine 1.0; $MgCl_2$ 0.5, supplemented with 3.0 mL of Tween 20 and the initial pH adjusted to 6.0. The inoculated flasks were incubated for four days at 28 °C under static conditions.

After the fermentation period, 100 mL of Tris-HCl buffer (0.05 M, pH 8.0) was aseptically added followed by shaking at 200 rpm for 1 h at room temperature. The fungal cultures were filtered through double-layered muslin cloth and the filtrate obtained was centrifuged for 10 min at 5 °C at 8000 ×*g*. The clear supernatant developed was termed crude-enzyme extract and was used for enzyme assay.

L-asparaginase and L-glutaminase assay

Nesslerization was used to calculate the activity of both L-asparaginase and L-glutaminase following the **Imada *et al*. (1973)** method. Briefly, the reaction mixture consisted of 0.1 mL of enzyme solution, 0.5 mL of 0.04 M L-asparagine or 0.04 M L-glutamine and 0.5 mL of 0.05 M Tris-HCl buffer (pH 8.0) was incubated at 37 °C for 30 min. The reaction was terminated by adding 0.5 mL of 1.5 M trichloroacetic acid (TCA), followed by centrifugation at 8000 ×*g* and the ammonia released was quantified colorimetrically by transferring 0.1 mL of the reaction into a clean test tube containing 0.2 mL of Nessler's reagent and 3.7 mL of distilled water. The mixture was incubated for 15 min at room temperature, and then the absorbance was measured at 450 nm. An analytical standard curve was prepared from ammonium chloride solutions to determine the amount of the released ammonia. One unit of enzyme activity (U) was described as the quantity of L-asparaginase that catalysed the formation of 1 µmol ammonia per min under standard assay conditions. The protein content was estimated according to **Bradford (1976)**. A calibration curve at 0.01-15.0 mg/L was made using BSA as standard. The specific activity is expressed as Unit/mg protein (U/mg).

Enzyme purification

Unless stated otherwise, all purification steps were performed at 5°C. Crude-enzyme filtrate was precipitated by gradual addition of cold ethanol (-20 °C) with gentle stirring until the final concentration of 50% saturation and maintained at 5 ºC for 12 h followed by centrifugation for 10 min at 4 ºC at 12, 000 ×*g*. Pellet formed was suspended in Tris-HCl buffer (0.05 M, pH 8.0), and dialyzed overnight toward distilled water at 5 ºC. The dialysate was loaded onto the gel filtration column of Sephadex G-100 (1.5 × 100 cm), pre-equilibrated with the same buffer. The bound protein was eluted using Tris-HCl buffer (0.05 M, pH 8.0) at 20 mL/h flow rate (each fraction contains 5 mL). L-asparaginase activity was evaluated using direct Nesslerization. Fractions with high activity were pooled, dialyzed toward distilled water for 12 h at 4 ºC, and concentrated by lyophilization. Lyophilized sample was further added to a Sephadex G-200 column (2.5 × 50 cm) that was previously equilibrated with Tris-HCl buffer (0.05 M, pH 8.0). Protein elution was constantly run at a flow rate adjusted to 15 mL/h using the same buffer and protein concentration was estimated in the eluent by measuring the absorbance at 280 nm (Cary-100 UV-Vis spectrophotometer, Germany). Active fractions with maximum activity of L-asparaginase were gathered, dialyzed, concentrated, and are used in the subsequent characterisation studies.

Molecular mass and homogeneity determination by electrophoresis

The concentrated sample was checked for purity using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) technique **(Laemmli 1970)** to estimate the molecular mass of the purified protein and ascertain homogeneity. Molecular mass markers starting from 11 to 245 kDa were used, and the molecular mass of purified L-asparaginase was estimated by plotting the logarithm of protein molecular mass markers versus relative protein mobility. The electrophoretic protein gel was run at 200 V for 40 min, then placed directly in the Coomassie Brillant Blue R-250 stain solution for 60 min, de-stained twice by 1.0% acetic acid solution, photographed while wet, dried and stored to determine the relative mobility of the purified enzyme.

Biochemical characterization of Trichoderma viride F2 L-asparaginase

Influence of pH and temperature on the activity and stability of L-asparaginase

Optimum pH of the purified enzyme was evaluated by measuring activity at different pH (from 3.0 to 10), using the following buffer solutions at 0.05 M concentration; sodium acetate buffer (3.0-6.0), phosphate buffer (6.0-7.0), Tris-HCl buffer (7.0-9.0) and carbonate-bicarbonate (9.0-10.0). The pH stability was assessed by pre-incubating the enzyme solutions (in the absence of the substrate) at various pH values (3.0-10.0) for 24 h at 5 °C. Residual activity of L-asparaginase was assayed as previously mentioned and the enzyme activity prior to incubation was considered as 100% activity. The optimal reaction temperature was calculated by performing the enzyme reaction at different temperatures (20 ºC - 80 ºC). Thermal stability was studied by pre-incubating the enzyme (without the substrate) at the desired temperature (50, 55, 60 ºC) for different time intervals (5 - 60 min), and then rapidly cooled down and the assay was performed under the optimum conditions (37 ºC and 8.0). The untreated enzyme was considered as the control (100%).

Substrate specificity

The substrate specificity of purified L-asparaginase was determined using a variety of natural substrates namely L-asparagine, D-asparagine, L-glutamine, D-glutamine, acrylamide and acetamide at 0.04 M final concentration. Relative activity was expressed as percentage ratio of enzyme activity estimated in terms of the various structural analogues of L-asparagine to enzyme activity with L-asparagine. The reaction rate was quantified in terms of the ammonia released.

Kinetic parameters

Different concentrations of the L-asparagine substrate (2.0 - 80 µmoles) were used to determine the kinetic parameters of the purified enzyme and the measurements were conducted at the optimum pH and temperature (8.0, 37 °C). As described by **Lineweaver and Burk (1934)**, the Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) were calculated as the absolute reciprocal values of the x and y-axis intercepts, respectively, of the linear regression curve.

Impact of different metal ions and chemical compounds on L-asparaginase activity

The effect of different metal ions and chemical compounds on the activity of L-asparaginase was evaluated by incubating each additive at 5.0 and 10 mM final concentration with the enzyme solution at room temperature for 1 h. Relative enzyme activity was calculated under the standard assay conditions by considering 100% activity of the non-incubated enzyme.

Mitigation of acrylamide formation in potatoes

Two grams of potato tubers (mashed in 0.05 M Tris-HCl buffer, pH 8.0) were applied to the purified L-asparaginase at 100 U/g of the potato. The mixture was incubated for 30 min at room temperature, and then the reaction was terminated by adding 0.5 mL TCA (1.5 M), followed by centrifugation at 8,000 rpm. The released ammonia was assessed by Nesslerization as previously described.

Antioxidant activity of *Trichoderma viride* F2 L-asparaginase

The antioxidant capacity of L-asparaginase was evaluated through its scavenging activity toward DPPH radicals. This assay is focused on the determination of the antioxidant's reduction potential against DPPH radical by calculating the reduction of its absorbance **(Prior *et al*. 2005)**. Briefly, 50 µL of different concentrations of L-asparaginase (25 - 125 µg/mL) as well as the reference standard (ascorbic acid) were taken and the volume was uniformly increased to 150 µL using methanol. Each sample was diluted with methanol up to 3.0 mL and 150 µL of freshly prepared DPPH solution (0.5 mM) in methanol was added; the mixtures were vigorously shaken and kept for 30 min in dark at room temperature. The absorbance was measured at 517 nm using methanol as a blank. A control reaction was consisted of 150 µL of DPPH and 3.0 mL methanol. The reduction in absorbance was considered as the anti-oxidant capacity of the purified L-asparaginase. The percentage of DPPH bleaching used for EC_{50} (the effective concentration needed to scavenge 50% of DPPH radicals) was determined as follows:

Radical scavenging activity (%) = $\frac{(A - B)}{A} \times 100$

Whereas, A: the absorbance of the negative control; B: the absorbance of the sample.

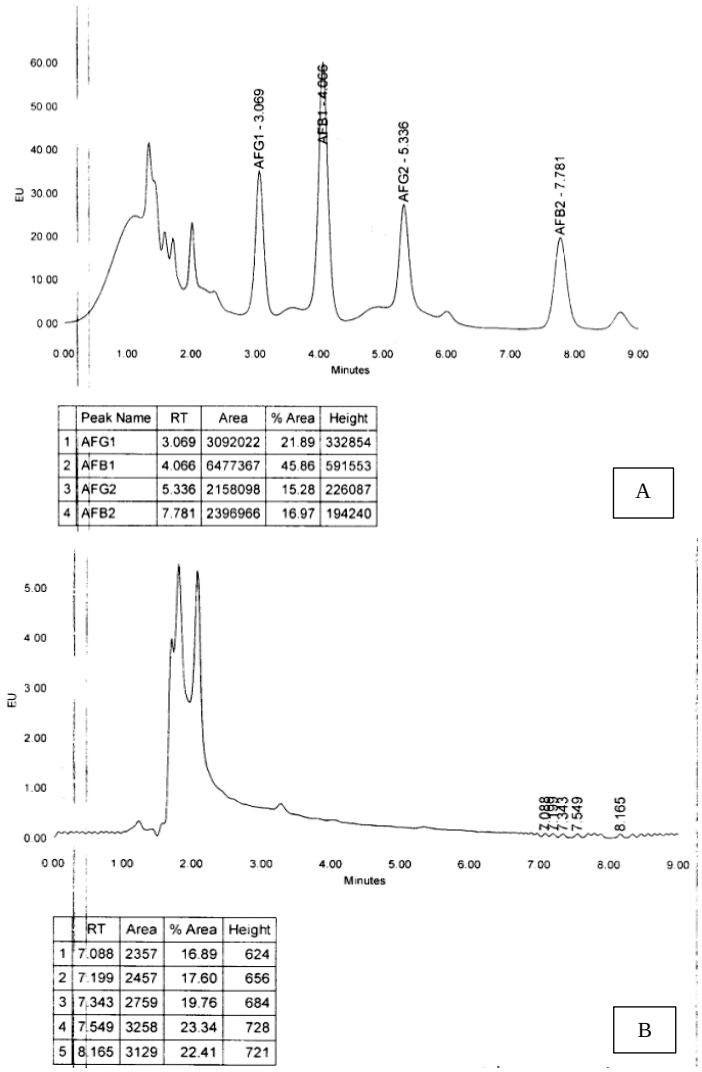
Statistical analysis

All experiments were performed in triplicate. Data were collected, revised, and entered to the statistical package for social science (SPSS) version 23; the quantitative parametric data were presented as mean and standard deviation (SD).

RESULTS AND DISCUSSION

Mycotoxins (Aflatoxins) detection

The toxicity test was conducted to verify the choice of the highest L-asparaginase producing fungal strain. Results reported in Figure 1 (A and B) clearly indicated that *Trichoderma viride* F2 was a non-mycotoxins producing fungus.



	Peak Name	RT	Area	% Area	Height
1	AFG1	3.069	3092022	21.89	332854
2	AFB1	4.066	6477367	45.86	591553
3	AFG2	5.336	2158098	15.28	226087
4	AFB2	7.781	2396966	16.97	194240

	RT	Area	% Area	Height
1	7.088	2357	16.89	624
2	7.199	2457	17.60	656
3	7.343	2759	19.76	684
4	7.549	3258	23.34	728
5	8.165	3129	22.41	721

Figure 1 A) standard determination of aflatoxins by HPLC; **B)** Auto scaled chromatogram for standard determination of afla-toxins by HPLC for *Trichoderma viride* F2

Purification of L-asparaginase from *Trichoderma viride* F2

The extracellular L-asparaginase was purified 36-folds with a final yield of 38.9% from *T. viride* F2 culture filtrate using a sequential multi-step process including ethanol precipitation (50% saturation), Sephadex G-100 gel filtration followed by Sephadex G-200 column chromatography. The elution profile on Sephadex G-200 gel filtration column showed four peaks for proteins and one peak for L-asparaginase activity. The peak of L-asparaginase activity was matched with the first peak of protein. While peaks II, III and IV showed lower protein content with no enzymatic activity (Fig. 2). The enzyme purification process was summarized in Table 1. The purified enzyme exhibited a specific activity of 688.1 U/mg protein, which was greater than that observed for L-asparaginases from *Mucor hiemalis* (69.43 U/mg) **(Thakur *et al.* 2014)** and *Penicillium* sp (13.97 U/mg) **(Patro and Gupta 2012)**. Comparatively, L-asparaginase was purified 106-fold from *Pseudomonas aureginosa* 50071, using ammonium sulphate precipitation, and gel filtration on Sephadex G-100 column followed by CM sephadex C-50 column **(EL-Bessoumy *et al.* 2004)**. **Dhevagi and Poorani (2006)** purified an intracellular L-asparaginase from *Streptomyces* sp. PDK2 up to 83-fold with a final yield of 2.18%, through gel filtration on Sephadex G-200.

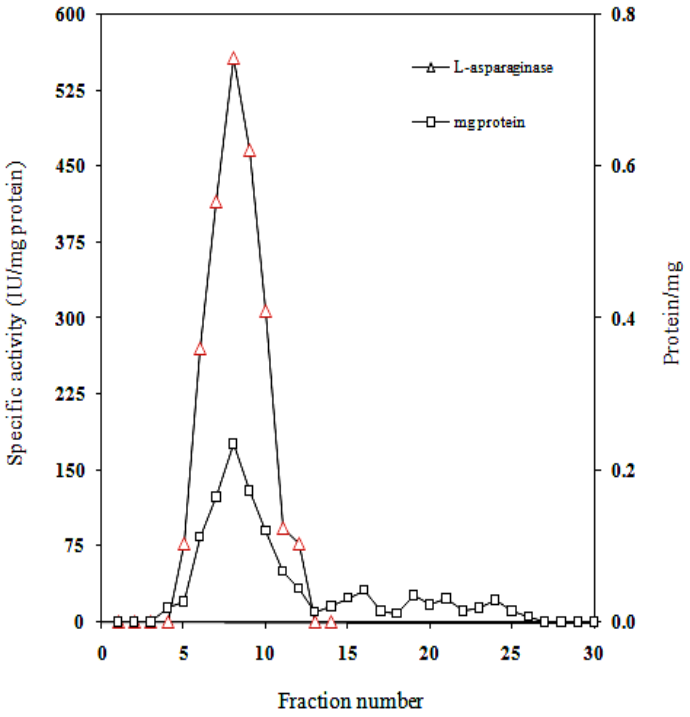


Figure 2 Purification profile of *Trichoderma viride* F2 L-asparaginase by gel filtration on Sephadex G-200 column

Molecular mass estimation by SDS-PAGE electrophoresis

The SDS-PAGE of the enzyme preparations formed from the sequential multi-step purification process revealed that the developed electrophoretic bands were enhanced from crude-enzyme extract to the final purification process (Fig. 3). It showed a single protein band with a molecular mass of 57 kDa, suggesting that the current *T. viride* F2 L-asparaginase was homogeneous and contained a single peptide chain. Molecular masses reported in several studies for L-asparaginases purified from various microorganisms such as *Penicillium brevicompactum* **(Elshafei *et al.* 2012)**, *Corynebacterium glutamicum* **(Savitri *et al.* 2003)**, *Streptomyces* sp. **(Dhevagi and Poorani 2006)**, and *S. albidoflavus* **(Narayana *et al.* 2008)** were 115 kDa, 80 kDa, 140 kDa and 116 kDa, respectively, while the molecular mass of *A. terreus* L-asparaginase was estimated to be 94 kDa **(Loureiro *et al.* 2012)**.

Table 1 Summary of steps employed in purification of L-asparaginase from *Trichoderma viride* F2

Purification step	Total activity (U)	Total protein (mg)	Sp. activity (U/mg protein)	Recovery (%)	Purification fold
Crude extract	4597±154	238±11.3	19.3	100	1.0
Ethanol precipitation (50%)	2863±58.2	31.4±2.15	91.2	62.3	4.7
Dialysis	2798±36.8	29.4±2.13	95.2	60.9	4.9
Sephadex G-100	2148±23.5	6.4±0.17	335.6	46.7	17.4
Sephadex G-200	1789±14.7	2.6±0.12	688.1	38.9	35.7

Data is expressed as mean ± SD of triplicates

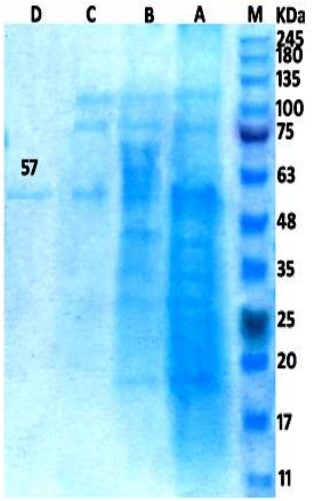


Figure 3 Electrophoretic analysis of *Trichoderma viride* F2 L-asparaginase. From right to left: *M*, standard protein markers of different molecular mass; *A*, crude enzyme extract; *B*, precipitation by cold ethanol; *C*, partial purified L-asparaginase on Sephadex G-100, *D*, purified L-asparaginase on Sephadex G-200

Biochemical characterization of *Trichoderma viride* F2 L-asparaginase

Impact of pH on the activity and stability of L-asparaginase

L-asparaginases from various sources differ widely in the optimal temperature and pH for optimum activity. Most of microbial L-asparaginase reported in the literature showed alkaline pH optima (8.0-10) **(Mahajan *et al*. 2014; Shanmugaprakash *et al*. 2015)**. In the current work, the purified *T. viride* F2 L-asparaginase was active and stable at pH values ranging from 7.0 to 9.0 but the maximum activity was exhibited at an intermediary pH of 7.5 (Fig. 4) compared with the pH values reported for L-asparaginase from *A. terreus* (pH 6.0) **(Balasubramanian *et al*. 2012)** and *A. niger* (pH 9.0) **(Akilandeswari *et al*. 2012)**. Maximal activity at physiological pH is considered as one of the necessities of L-asparaginase for therapeutic efficacy; therefore the current *T. viride* F2 L-asparaginase will be useful since 100% of its activity maintained at pH 7.5. Moreover, this purified enzyme showed high stability at alkaline pH relative to acidic pH, as it retained more than 90% of its initial activity at pH 7.0-9.5 after 24 h of incubation at 5 °C. In addition, at pH 10, the enzyme still retained 88% of its initial activity (Table 2). Likewise, L-asparaginases purified from *S. marcescens* and actinomycetes were found to have a wide pH optimum ranging from pH 5 to 9 **(Khamna *et al*. 2009; Novak and Phillips 1974)**. While L-asparaginase of *A. niger* AK10 worked optimally at pH 8.6 and was more stable when incubated at pH 8.0-8.6 **(Dharmsthiti and Luechai 2011)**. In addition, the optimum activity of *P. aeruginosa* 50071 L-asparaginase and *Streptomyces* sp. PDK7 L-asparaginase was investigated at pH 9 and 8 **(El-Bessoumy *et al*. 2004; Dhevagi and Poorani 2006)**. A membrane bound L-asparaginase purified from *Tetrahymena pyriformis* was found to act optimally at pH 9.6 as reported by **Triantafillou *et al*. (1988)**.

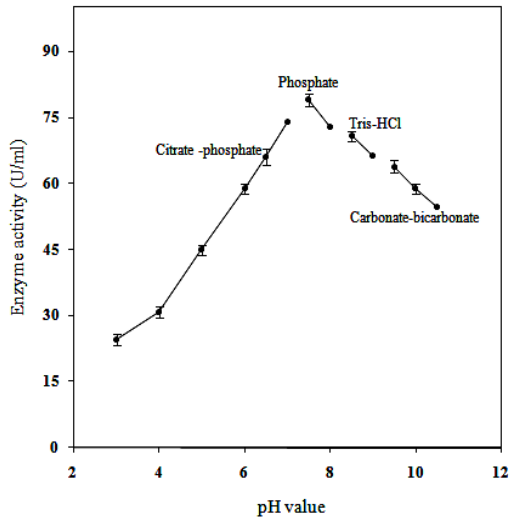


Figure 4 *Trichoderma viride* F2 L-asparaginase activity as a function of pH value of the reaction mixture

Table 2 Determination of pH stability of the purified *Trichoderma viride* F2 L-asparaginase

Buffer (0.05 M)	pH value	Relative activity (%)
Citrate-phosphate	3.0	53.3±2.13
	4.0	54.2±1.87
	5.0	58.3±2.12
	6.0	68.4±1.88
	6.5	76.5±1.24
	7.0	95.8±3.12
Phosphate	7.0	97.3±2.89
	7.5	100±1.02
	8.0	100±1.09
Tris-HCl	8.0	100±1.05
	8.5	94.7±0.89
	9.0	93.8±1.32
Carbonate-bicarbonate	9.0	92.4±1.43
	9.5	91.7±1.22
	10.0	88.2±0.91

Data is expressed as mean ± SD of triplicates

Influence of temperature on the activity and stability of L-asparaginase

The reaction rate of *T. viride* F2 L-asparaginase was determined at different temperatures (20 ºC - 80 ºC). As reported in Fig. 5, the purified enzyme was active at temperatures ranging from 37 °C to 45 °C; however at higher temperatures the enzyme activity declined. In addition, *T. viride* F2 L-asparaginase was found to be stable and retained 100% of its original activity at temperatures up to 45 ºC (Fig. 6), while at 50 ºC the enzyme maintained 71% and 39% of its activity after 30 and 60 min of incubation, respectively. This finding suggests that the purified *T. viride* F2 L-asparaginase can be easily adopted and act normally in the human body's internal environment for complete removal of asparagine from the body. Similarly, the maximal activity of L-asparaginases purified from *Pseudomonas aeruginosa* 50071 and *Pectobacterium carotovorum* has been observed at 37 ºC **(El-Bessoumy *et al*. 2004; Shanmugaprakash *et al*. 2015)**. While that of *E. carotovora* and *Streptobacillus* sp. L-asparaginases were detected at 35 ºC **(Kamble *et al*. 2006; Makky *et al*. 2013)**. On the other hand, L-asparaginases of *Chrombacteriaceae* and *Proteus vulgaris* were reported at 20 ºC and 57 ºC, respectively **(Roberts *et al*. 1972; Tosa *et al*. 1972)**.

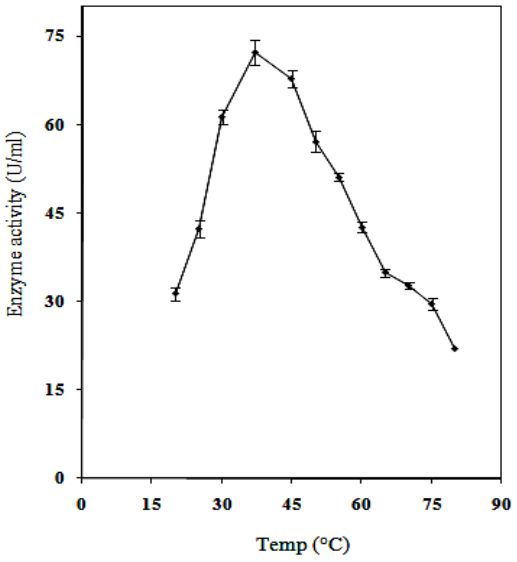


Figure 5 Effect of temperature on L-asparaginase activity

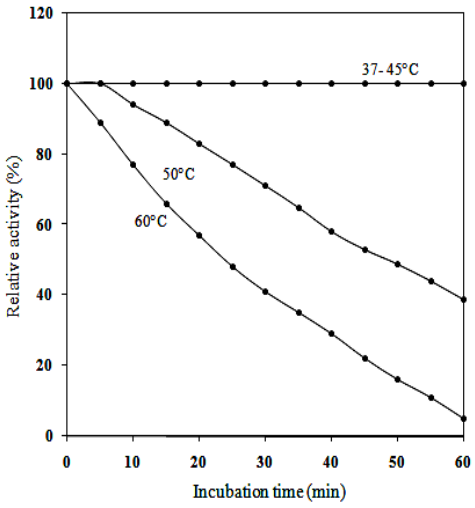


Figure 6 Heat stability and inactivation kinetics

Effect of inhibitors/activators on L-asparaginase activity

The activity of *T. viride* F2 L-asparaginase was determined in the presence of different compounds (Table 3). Among the salts tested, univalent cations, Na^+ and K^+ acting somewhat as activators and enhanced L-asparaginase activity by 145.7% and 163.5%, respectively, whereas, bivalent cations Ca^{2+} slightly enhanced the activity by 112%. While a considerable loss of L-asparaginase activity was reported with Ag^+ and Fe^{+3}. The metal chelating agent, EDTA, showed no effect on the activity, suggesting that the current L-asparaginase is not a metalloenzyme. Inhibition of the activity in the presence of Hg^{2+}, Cd^{2+}, and Zn^{2+} may be an attributable to the important vicinal sulfhydryl groups (-SH) for the productive catalysis. Moreover, improvement in the activity of the current L-asparaginase with reducing agents such as reduced glutathione (GSH) and mercaptoethanol (2-ME) and inhibition with thiol group blocking agent iodoacetamide (IA) provided additional evidence of the role of SH groups in the enzyme catalytic activity. Reduced glutathione acts as an important and effective molecule associated with redox changes. Glutathione has been reported to potentiate *Cylindrocarpon obtusisporum* and *E. carotovora* L-asparaginase activity **(Raha *et al*. 1990; Warangkar and Khobragade 2010)**.

Table 3 Effect of different metal ions and chemical compounds on *Trichoderma viride* F2 L-asparaginase activity

Activator/inhibitor	Relative activity (%)	
	5 mM	10 mM
Control (without metal ion)	100±0.87	100±1.08
NaCl	125.9±1.02	145.7±1.34
KCl	138.5±1.23	163.5±2.35
AgCl	51.3±0.76	38.4±0.54
$ZnCl_2$	64.8±0.69	40.6±0.78
$CaCl_2$	112.3±1.09	105.6±0.92
$BaCl_2$	99.2±0.98	76.4±0.56
$HgCl_2$	60.1±1.21	32.9±0.32
$CoCl_2$	91.2±0.87	77.6±0.56
$CuCl_2$	73.8±0.45	57.6±0.23
$CdCl_2$	63.6±0.92	37.8±0.43
$PbCl_2$	92.6±1.03	80.2±0.91
$FeCl_3$	69.5±0.21	43.5±0.32
EDTA	101.2±0.65	105.4±1.34
Iodoacetamide	53.2±1.23	21.9±0.41
β-Mercaptoethanol	118.4±0.87	108.7±0.18
R-Glutathione	114.5±0.76	103.1±0.87

Data is expressed as mean ± SD of triplicates

Substrate specificity of Trichoderma viride F2 L-asparaginase

A variety of substrates of asparagine analogues were used to assess the substrate specificity of *T. viride* F2 L-asparaginase and data were summarized in Table 4. The activity using L-asparagine as a substrate was considered as 100% activity. The purified L-asparaginase was found to be not active toward the tested substrates with the exception of acrylamide which was quite close to that of L-asparagine. This property makes L-asparaginase of *T. viride* F2 potentially very useful in food applications. D-asparagine and acetamide were hydrolyzed by 15.6% and 11.8%, respectively. No activity was reported with L-glutamine as substrate, indicating that the purified enzyme was free from L-glutaminase activity. Contamination of L-asparaginase with L-glutaminase activity has been reported to cause adverse effects during cancer treatment **(Manna *et al*. 1995)**. Accordingly, these findings suggest that *T. viride* F2 L-asparaginase is very specific to its natural substrate L-asparagine. Similar findings were reported by **Kumar *et al*. (2011)** other than that the enzyme showed very low activity toward D-glutamine, D-asparatic acid, L-asparagine-t-butyl ester HCl, succinamic acid and N-acetyl L-asparagine.

Table 4 Substrate specificity of the purified *Trichoderma viride* F2 L-asparaginase

Substrate (0.04 M)	Relative activity (%)
L-asparagine	100±0.87
D-asparagine	15.6±0.19
L-glutamine	0.0
D-glutamine	0.0
NAD	0.0
Acetamide	11.8±0.09
Acrylamide	98.4±1.02

Data is expressed as mean ± SD of triplicates

Kinetic parameters

The purified L-asparaginase demonstrated Michaelis–Menten kinetics at relatively low substrate concentrations and the values of K_m and V_{max} for L-asparagine were found to be 1.25 mM and 71.3 U/ml, respectively (Fig. 7). This finding suggests that L-asparaginase has a high affinity against its natural substrate, which may be related to its degree of tumour efficacy. **Chang and Franden (1981)** reported higher K_m values of 6.6 and 7.0 mM for L-asparaginase from *Lupinus arboreus* and *Lupinus angustifolius*, respectively. On the contrary, a lower K_m of 0.058 mM was investigated for *Erwinia chrysanthemi* L-asparaginase **(Kotzia and Labrou 2007)**. In addition, higher K_m values of 3.5 and 7.14 mM were reported for L-asparaginases purified from *Escherichia coli* and *Erwinia carotovora*, respectively **(Kumar *et al*. 2010)**.

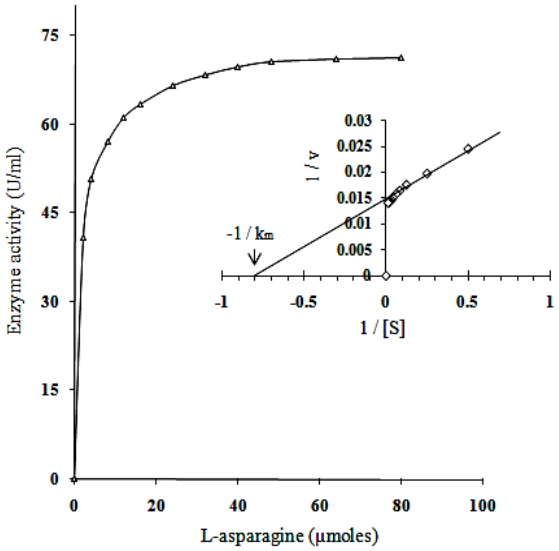


Figure 7 Determination of K_m of the purified *Trichoderma viride* F2L-asparaginase for L-asparagine by non-linear regression analysis of experimental steady state data. A plot of reaction velocity (V) versus substrate concentration (2.0 - 80 µmol) fitted to the Michaelis–Menten equation

Mitigation of acrylamide formation in potatoes

The release of ammonia of about12.4 µmoles/mL was observed when the purified *T. viride* F2 L-asparaginase was tested for its effect on L-asparagine present in potato, suggesting the conversion of L-asparagine to L-aspartic acid. This outcome makes *T. viride* F2 L-asparaginase a potential candidate in reducing acrylamide level of starchy fried food.

DPPH radical scavenging activity

DPPH is a stable dark violet radical owing to its unpaired electron. In the presence of an antioxidant radical scavenger that can donate an electron to DPPH, the dark violet color is decolorized to a pale yellow non-radical color **(Naveena *et al*. 2012)**. In this study, L-asparaginase was found to demonstrate a great DPPH radical scavenging that was improved with rising concentration. The IC_{50} values for L-asparaginase and ascorbic acid (standard) were estimated to be 57.4 and 66.1 µg/mL, respectively, as shown in Figure 8.

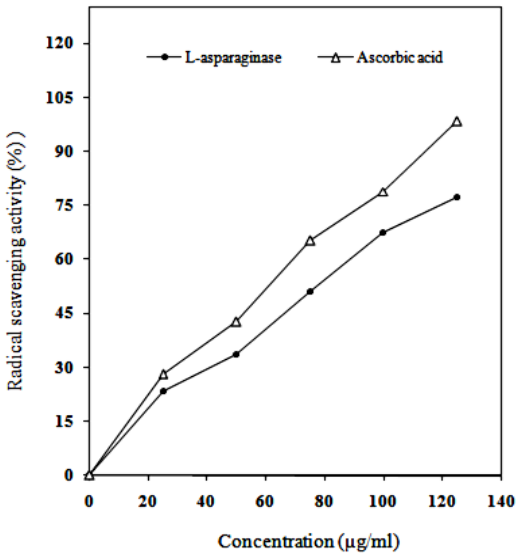


Figure 8 DPPH scavenging activity of the purified *Trichoderma viride* F2 L-asparaginase

CONCLUSION

This study explores the purification and biochemical characterization of extracellular glutaminase-free L-asparaginase produced by a non-mycotoxins producing terrestrial fungus *T. viride via* SSF using agro-industrial residues as substrate. Biochemical studies on the purified enzyme indicated that this enzyme has a good activity at the physiological pH, stability at elevated temperatures, high affinity towards its natural substrate L-asparagine (low K_m value)…etc that encouraged us to suggest this *T. viride* F2 L-asparaginase as an antitumor drug and worth further investigations of its proper utilization. In addition, data reported in this study revealed that the current L-asparaginase hydrolyzed L-asparagine present in potatoes to L-aspartic acid ammonia, making it a promising candidate for food industry to decrease the level of acrylamide in starchy fried food.

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