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Purification and Characterization of a New L-Methioninase from Solid Cultures of *Aspergillus flavipes*

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L-Methioninase was purified to electrophoretic homogeneity from cultures of *Aspergillus flavipes* using anion-exchange and gel filtration chromatography by 12.1 fold compared to the crude enzyme preparation. The purified enzyme had a molecular mass of 47 kDa under denaturing conditions and an isoelectric point of 5.8 with no structural glycosyl residues. The enzyme had optimum activity at pH 7.8 and pH stability from 6.8-8.0 at 35°C. The enzyme appeared to be catalytically stable below 40°C. The enzyme activity was strongly inhibited by DL-propargylglycine, hydroxylamine, PMSF, 2-mercaptoethanol, Hg^{2+}, Cu^{2+}, and Fe^{2+}, with slight inhibition by Triton $X\text{-}_{100}$. *A. flavipes* L-methioninase has a higher catalytic affinity towards L-methionine (Km, 6.5 mM and Kcat, 14.1 S^{-1}) followed by a relative demethiolating activity to L-homocysteine (Km, 12 mM and Kcat, 9.3 S^{-1}). The enzyme has two absorption maxima at 280 and 420 nm, typical of other PLP-enzymes. Apo-L-methioninase has the ability to reconstitute its structural catalytic state completely upon addition of 0.15 mM PLP. L-Methioninase has neither an appreciable effect on liver function, platelet aggregation, nor hemolysis of human blood. The purified L-methioninase from solid cultures of *A. flavipes* displayed unique biochemical and catalytic properties over the currently applied *Pseudomonad* enzyme.

***Keywords*:** L-methioninase, *Aspergillus flavipes*, solid state fermentation, PLP enzyme properties, cytotoxicity

L-Methioninase (E.C 4.4.1.11) is a pyridoxal phosphate-dependent enzyme that catalyzes the direct α and β-elimination of L-methionine to α-ketobutyrate, methanethiol, and ammonia (Tanaka *et al*., 1976). The enzyme has the ability to catalyze α and β-elimination reactions of cysteine and their analogues (Tanaka *et al*., 1985).

The enzyme has received much attention, since it was reported as a potent anticancer agent against various types of tumor cell lines (Tan *et al*., 1998). Physiologically, normal cells have the ability to grow on homocysteine, instead of methionine, due to their active methionine synthase (Mecham *et al*., 1983). Unlike normal cells, tumor cells devoid of active methionine synthase thus depend on external methionine supplementation from the diet (Hoffman, 1984). Methionine-dependency was reported as a physiological character for colon, kidney, prostate, melanoma, and fibrosarcoma tumor cells (Miki *et al*., 2000; Yamamoto *et al*., 2003).

L-Methioninase has been purified and characterized from various bacterial species including *Pseudomonas putida* (Tanaka *et al*., 1977), *Aeromonas* sp. (Tanaka *et al*., 1985), *Citrobacter freundii* (Manukhov *et al*., 2005), *Brevibacterium linens* (Amarita *et al*., 2004), *Lactococcus lactis* (Martinez-Cuesta *et al*., 2006), and *Clostridium sporogenes* (Krishnaveni *et al*., 2009). Also, the enzyme was purified from *Arabidopsis thaliana* (Rebeille *et al*., 2006). Despite the low therapeutic efficiency of bacterial L-methioninase, due to a high immunogenicity and rapid plasma clearance (Kudou *et al*., 2007), the purified enzyme has not been comprehensively characterized from a fungal source. Unlike bacterial L-methioninase, the eukaryotic/fungal enzyme may display a lower immunogenic reaction during the course of tumor therapy, which may be associated with the structural competence of the human immune system. A few studies on the partial characterization of L-methioninase from fungi including *Penicillium* sp. (Tsugo and Matsuoka, 1962), *Aspergillus* sp RS-1a (Ruiz-Herrera and Starkey, 1969), *Humicola fuscoatra* (Faleev *et al*., 1996), and *A. flavipes* (El-Sayed, 2009b) have been reported.

Searching for new microorganisms with superior enzyme productivity is reasonable due to the broad therapeutic applications of L-methioninase against various types of tumors. Fungi appear to be the potent eukaryotes for enzyme production not only from the pharmacokinetic point of view but also from the expense of fermentation conditions. Solid state fermentation is a selective condition for fungal growth and enzyme production (El-Sayed, 2009a, 2009b). Consequently, production and optimization of L-methioninase by *A. flavipes* under solid state fermentation using chicken feather as a substrate was conducted by our lab (El-Sayed, 2009b).

Therefore, this study was extended to complete our previous study by purifying and comprehensively characterizing L-methioninase from solid cultures of *A. flavipes* using chicken feather-solid fermented medium.

Materials and Methods

Materials

L-Methionine, DL-homocysteine, L-cysteine, L-cystine, L-asparagine, L-glutamine, L-arginine, L-phenylalanine, phenylmethylsulfonyl fluoride (PMSF), *N*-acetylglucosamine, pyridoxal L-phosphate, and

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DL-propargylglycine were purchased from Sigma-Aldrich Co. (USA). Folin reagent, L-lysine, and hydroxylamine were obtained from LOBA Chemie (India). Sephadex G_{100}, G_{200}, and DEAE-cellulose were purchased from Pharmacia Biotechnology (Sweden). Chicken feathers were collected from the local poultry breeding laboratories (Egypt), washed several times with commercial detergents, dried, and used as a substrate for solid state fermentation. All other chemicals were of analytical grade.

Fungal strain and growth conditions

A. flavipes (Bain and Sart) Thom and Church was selected as a potent L-methioninase producer from our preliminary studies (Khalaf and El-Sayed, 2009). Chicken feathers were used as a solid substrate for enzyme production. *A. flavipes* was grown on optimized solid state fermentation medium (El-Sayed, 2009b) to produce L-methioninase. After inoculating the solid medium using *A. flavipes* conidial suspensions (7-day-old cultures), the solid cultures were incubated at 28± 1°C for 10 days under static conditions.

Extraction of L-methioninase

After incubating the solid *A. flavipes* cultures, the crude enzyme was extracted by the simple contact method using a pH 7.0 potassium phosphate buffer (El-Sayed, 2009b). The clear supernatant was used as the crude enzyme preparation for the subsequent steps.

Purification of L-methioninase

The crude enzyme was fractionated using ammonium sulfate. The calculated amount of ammonium sulfate was added gradually to the supernatant, with stirring, to obtain 50-70% saturation. After 12 h at 4°C, the precipitate was collected by centrifugation at 5,000 rpm for 15 min, resuspended in a minimum volume of 50 mM potassium phosphate buffer (pH 7.0) containing 20 μM pyridoxal phosphate and 1 mM EDTA, and then dialyzed against the same buffer. The dialysis step was repeated until the complete removal of ammonium sulfate, as verified by barium chloride.

Anion exchange chromatography

The dialysate was loaded onto a DEAE-cellulose column (2×30 cm), which had been washed and equilibrated with potassium phosphate buffer (pH 7.0) containing 20 μM pyridoxal phosphate. After equilibration, the L-methioninase fractions were eluted with a linear gradient of sodium chloride (50 mM-1 M) in the same buffer. The active fractions were pooled, concentrated, and collected prior to the next purification step.

Gel filtration chromatography

The active fractions from the DEAE-cellulose column were further purified with a Sephadex G_{200} column. After equilibration with the same buffer, the enzyme was loaded on the top of the column. The enzyme fractions were eluted with the same buffer containing 20 μM pyridoxal phosphate at a flow rate 0.5 ml/4-5 min. The enzyme activity and their protein were assayed.

The active L-methioninase fractions were pooled, combined, and subsequently applied to the top of a Sephadex G_{100} column, pre-equilibrated with the same buffer. The activity and enzyme concentration were determined after elution of the enzyme fractions.

L-Methioninase assay

The demethiolating activity of L-methioninase was expressed by the amount of methanthiol from L-methionine as adopted by Laakso and Nurmikko (1976). The reaction mixture contained 20 mM L-methionine in potassium phosphate buffer (pH 7), 0.1 mM pyridoxal phosphate, 0.25 mM 5, 5-dithio-bis-2-nitrobenzoic acid (DTNB) in a final volume of 1.0 ml. Enzyme and substrate blanks were prepared separately. After a 1-h incubation of the mixture at 37°C, the increase in absorbance of the developing yellow color was measured at 420 nm. The demethiolating activity of the enzyme was expressed as the amount of methanethiol from a standard curve with different methanethiol concentrations and DTNB, under assay conditions. One unit (U) of L-methioninase was expressed as the amount of enzyme that releases 1 μmol of methanethiol per min under optimal assay conditions. Specific activity of the enzyme was represented by the mean activity (U) per mg enzyme protein.

The deaminating activity of L-methioninase was assessed by the Nesslerization method (Khalaf and El-Sayed, 2009). One unit (U) of enzyme activity was expressed as the amount of enzyme that releases one μmol of ammonia per minute under optimal assay conditions.

Protein concentration

The enzyme protein content was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as the standard.

SDS-polyacrylamide gel electrophoresis

The homogeneity of purified L-methioninase was checked using SDS-PAGE (Laemmli, 1970). The protein sample (50 μl) was boiled in dissociation buffer containing 50 mM Tris-HCl (pH 7.0), 1.0% glycerol, 2% (w/v) SDS, 5% 2-mercaptoethanol, and 0.1% bromophenol blue. After running, the gel was stained with Coomassie Brilliant Blue R-250 for 12 h under shaking (80 rpm). After decolorizing the gel with methanol-acetic acid, the bands were visualized using a gel documentation system. The homogeneity of the purified enzyme was checked, and the molecular mass was determined from the calibration against protein markers. The markers were: myosin (210.0 kDa), phosphorylase B (120.0 kDa), bovine serum albumin (84.0 kDa), ovalbumin (60.0 kDa), carbonic anhydrase (39.0 kDa), trypsin inhibitor (28.0 kDa), and lysozyme (18.3 kDa).

Determination of L-methioninase pI

The isoelectric point for L-methioninase was determined as described by Kantardjieff and Rupp (2004) with slight modifications. The enzyme preparation was incubated at different pHs (5.4-7.0) using potassium phosphate buffer at 4°C. After a 12 h incubation, the enzyme was precipitated by centrifugation at 10,000 rpm for 10 min. The precipitated protein was measured quantitatively using Folin reagent. The isoelectric point was expressed as the pH range at which the maximum enzyme precipitation occurred.

Absorption spectrum

The absorption spectrum of purified L-methioninase was determined in the range of 200 to 600 nm using a Shimadzu uv/vis scanning spectrophotometer (UV-1600; Shimadzu, Japan). The enzyme concentration was 500 μg/ml in potassium phosphate buffer (pH 7.8). The spectra were measured for the purified holo-methioninase against a blank of potassium phosphate buffer (pH 7.8) containing pyridoxal phosphate.

Dissociation and reconstitution of enzyme-PLP

Apo-L-methioninase was prepared by incubating the enzyme with 10 mM hydroxylamine in potassium phosphate buffer (pH 7.8) for 1 h at 4°C, followed by dialysis against three changes of the same

buffer for 5 h at 4°C. The apo-enzyme spectra were recorded under the same conditions. The demethiolating activity of the enzyme was measured as described above. The reconstitution of the catalytic activity of the enzyme was monitored by adding different concentrations of pyridoxal phosphate (0.04, 0.08, 0.1, 0.15, and 0.2 mM final concentration). The demethiolating activity and spectral analysis for the recovered holo-enzyme were determined for each treatment.

Determination of the glycosyl-residues of L-methioninase

The structural glycosyl-moieties of the purified enzyme were determined by the Amplex Red Glucose Oxidase Assay kit (Amplex, Invitrogen, UK) for glycoprotein estimation (Kinoshita *et al*., 2000). Briefly, the enzyme preparation (250 µl) was incubated with Amplex Red reagent (100 µM), glucose oxidase (4 U/ml), and horseradish peroxidase (0.5 U/ml) for 30 min at 37°C. After the incubation, resorufin fluorescence was quantified at an excitation wavelength of 530 nm and a detection wavelength of 590 nm. The amount of glucose was determined from a fluorescence calibration curve using different glucose concentrations.

Biochemical properties of L-methioninase

The optimum pH for L-methioninase activity was determined using 50 mM citrate-phosphate buffer (pH 3.0-6.5), 50 mM potassium phosphate buffer (pH 5.2-8.0), and 50 mM glycine-NaOH buffer (pH 8.0-10.6). After incubating each reaction mixture for 1 h, enzymatic activity was stopped and assayed by the standard method. The pH stability of the purified enzyme was determined by preincubating the enzyme at pHs ranging from 3.8 to 10.4 for 2 h at 4°C and then measuring the residual activity by the normal assay method.

The effect of reaction temperature on the demethiolating activity of L-methioninase was determined by incubating the reaction mixture at different temperatures (20-60°C) under optimal assay conditions. The thermal stability of the purified enzyme was determined by preincubating the enzyme in potassium phosphate buffer (pH 7.0) at various temperatures (20-60°C) for different times (30-150 min) and measuring the residual demethiolating activity. The *Tm* value is the temperature at which enzyme retains 50% of its initial activity after heating for 60 min.

The influence of some ions and organic compounds on the activity of *A. flavipes* L-methioninase was evaluated by preincubating the enzyme without substrate but with the chloride salts of metal ions such as Na^{+}, K^{+}, Se^{2+}, Cd^{2+}, Fe^{+2}, Mn^{2+}, Ca^{2+}, Co^{2+}, Zn^{2+}, Hg^{2+}, Li^{2+}, Ni^{2+}, and Cu^{2+} in addition to glucose, fructose, and potassium oxalate. The inhibitory effect of DL-propargylglycine, EDTA, 2-mercaptoethanol, dithiothritol, hydroxylamine, guanidine-HCl, PMSF, DMSO, sodium dodecyl sulfate, Triton X-100, and Tween 80 on enzyme activity was also assessed. The enzyme was incubated with each compound for 20 min, before adding L-methionine as the substrate (20 mM). The reaction was incubated for 1 h at 37°C. The residual activity of the enzyme was expressed as the amount of methanethiol released under optimum assay conditions.

The specificity of the purified *A. flavipes* L-methioninase towards various substrates was evaluated. The ability of the purified enzyme to catalyze the elimination reactions of amino acids and their analogues was evaluated separately towards DL-homocysteine, L-cysteine, L-cystine, L-tyrosine, D-valine, L-lysine, L-glutamine, L-glutamic acid, L-asparagine, L-aspartic acid, D-alanine, L-arginine, L-phenylalanine, and D-glycine, using L-methionine as the standard substrate. The enzyme activity against sulfur amino acids (DL-homocysteine, L-cysteine, and L-cystine) was assessed by the normal demethiolating assay, as described above. Unlike sulfur amino acids, the activity towards all other amino acids except L-tyrosine and L-phenylalanine was determined with the standard deaminating assay, as described above. Tyrosine hydrolyzing activity was measured as oxidase by formation (Krishnaveni *et al*., 2009). L-Phenylalanine hydrolyzing activity was expressed as the amount of cinnamic acid at 290 nm (Fritz *et al*., 1976). Also, the deacetylating activity of the enzyme towards *N*-acetylglucosamine was evaluated by the method of Ride and Drysdale (1972). The proteolytic activity of L-methioninase was quantified (Ledoux and Lomy, 1986) using casein as a substrate. L-Methioninase activity was determined by incubating the enzyme with 20 mM of each substrate in potassium phosphate buffer (pH 7.8) containing 0.1 mM pyridoxal phosphate, in a total volume of 1.0 ml. The enzyme activity was measured by the corresponding method after the incubation.

Kinetic studies

The kinetic parameters of purified L-methioninase towards the various selected substrates were evaluated in potassium phosphate buffer (pH 7.0) at 30°C using different concentrations of each substrate (10-100 mM). The activity was determined regarding the demethiolation of each substrate. The kinetic parameters such as the Michalis-Menten constant (Km) and maximum velocity (Vmax) were calculated from a Lineweaver-Burk plot. The catalytic efficiency (Kcat) of the enzyme for each substrate was expressed by the specific activity per mole of the enzyme.

Cytotoxicity of *A. flavipes* L-methioninase

Biochemical changes *in vivo*: Male Swiss albino mice (6~8-weeks-old) were injected intraperitoneally with 0.5 ml of different enzyme doses (2.0, 5.0, 10.0, and 14.6 U/mg protein) and incubated for 5 days under controlled conditions. Blood was collected in 2% EDTA and centrifuged at 5,000 rpm for 10 min. The activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Reitman and Frankel, 1957) were determined in blood plasma. Hemoglobin and erythrocyte concentrations were estimated according to the method of Birt (1967). Controls (without treatments) and blanks (injected with potassium phosphate buffer) were used. Triplicates were used

Table 1. Purification profile of *Aspergillus flavipes* L-methioninase

Purification Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Yield (100%)	Purification fold
Crude enzyme	1470	1766	1.21	100	1
Precipitated by 50-70% NH_4SO_4	576	1305.6	2.26	73.9	1.86
DEAE-Cellulose	229.8	920	5.89	52.1	4.8
Sephadex G_{200}	82.2	786	9.30	44.5	7.6
Sephadex G_{100}	48	704	14.6	39.8	12.1

The amount of protein is 2.2 mg/ml for the final purified enzyme.
Activity of L-methioninase is 32.2 U/ml.

for each group.

Platelet aggregation: A platelet aggregation assay was conducted following the turbidimetric method of Wei *et al.* (2007). A human blood specimen was collected in 0.4% sodium citrate. After centrifugation at 8000 rpm for 5 min at room temperature, the platelet-rich plasma (350 µl) was collected, injected with 50 µl of enzyme (14.0 U/mg protein), and incubated for 20 min at room temperature. Platelet aggregation was monitored with a blood Lumi-Aggregometer (Chrono-Log Corp., USA).

Hemolytic activity: Hemolytic activity of the purified *A. flavipes* L-methioninase was evaluated using a blood agar assay (Tay *et al*., 1995). Briefly, 5 ml of human blood was added to sterile agar-agar (2.0%) in potassium phosphate buffer. After pouring and solidification of the medium, 4.0 mm diameter wells were made, pipetted with 200 µl enzyme (14.6 U/mg), and incubated overnight at room temperature. Then, the hemolytic activity of the enzyme appeared as a halo zone.

Statistical analysis: All experiments were conducted in triplicate. The data are presented as the average of triplicates±SDs.

Results

Purification of L-methioninase

A crude preparation of L-methioninase was obtained from the solid cultures of feather-grown *A. flavipes* under previously optimized solid state fermentative conditions (El-Sayed, 2009b). L-Methioninase was fractionated from the solid *A. flavipes* cultures using ammonium sulfate (50-70%) followed by anion-exchange chromatography and gel filtration chromatography. From the purification profile (Table 1), the specific activity of L-methioninase increased from 1.21 to 2.26 U/mg following salting out, with a 73.9% yield. By applying NaCl gradient concentrations of 50 mM-1 M to elute the L-methioninase from the DEAE-cellulose column, the highest enzyme activity (5.89 U/mg protein) was obtained using 125 mM NaCl, resulting in a single sharp peak (Fig. 1). The specific activity of L-methioninase increased 4.8 fold, with a 52.1% recovery from DEAE-cellulose. Subsequently, the peaked fractions were gathered and applied to the top of a Sephadex G_{200} column. The enzyme was eluted using potassium phosphate buffer (pH 7.0) containing 20 µM pyridoxal phosphate. A single peak of L-methioninase with a specific activity 9.3 U/mg protein (7.6 fold) and 44.5% yield was recovered (data not shown). Moreover, using Sephadex G_{100} and the same elution buffer, L-methioninase specific activity increased to 14.6 U/mg protein (Fig. 2). From the overall purification profile (Table 1), the enzyme was purified by 12.1 fold with a 39.8% yield, using the three chromatographic steps compared to the crude enzyme.

Molecular weight and subunit structure

The molecular weight of the purified L-methioninase was 47 kDa under SDS-PAGE denaturing conditions. The sequential purification steps appeared from the gel-electrophoresis profile (Fig. 3). The appearance of L-methioninase as a single band under denaturing conditions ensured their homogeneity.

Isoelectric point for L-methioninase (pI)

The isoelectric point for *A. flavipes* L-methioninase was 5.8 based on the pH-precipitation profile (Fig. 4).

Absorption spectra of L-methioninase

The native purified holoenzyme displayed absorption maxima at 280 nm and at 420 nm in potassium phosphate buffer (pH 7.8) containing 20 µM pyridoxal phosphate. The holoenzyme was preincubated with 10 mM hydroxylamine for 20 min and then dialyzed against potassium phosphate buffer. The released apo-methioninase exhibited a maxima at 280 nm, with complete disappearance of the 420 nm peak, which may have been due to the dissociation of pyridoxal phosphate. The holoenzyme was reactivated by adding various concentrations of pyridoxal phosphate as shown by the absorption spectra profile and activity measurement (Fig. 5). The rate of enzyme reconstitution was expressed by the ratio of absorbance A280/

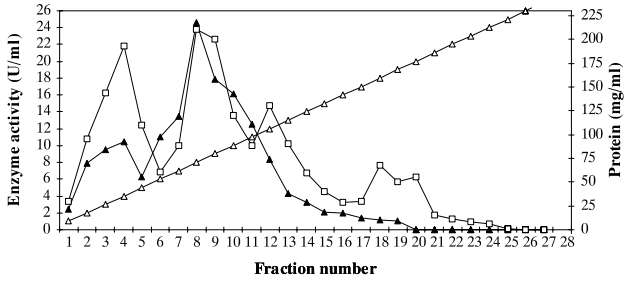


Fig. 1. DEAE-Cellulose fractionation of *A. flavipes* L-methioninase. Enzyme activity (▲) and protein content (□). The enzyme fractions were eluted by gradient NaCl (50 mM-1 M) in potassium phosphate buffer (pH 7.0).

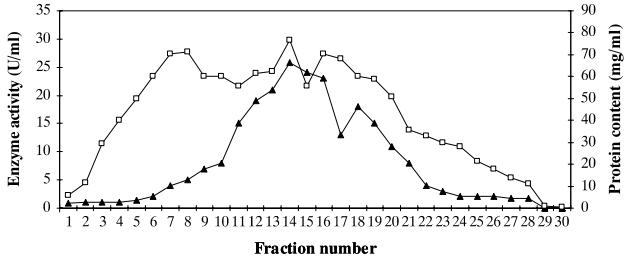


Fig. 2. Sephadex G $_{100}$ fractionation of *A. flavipes* L-methioninase. Enzyme activity (▲) and protein content (□).

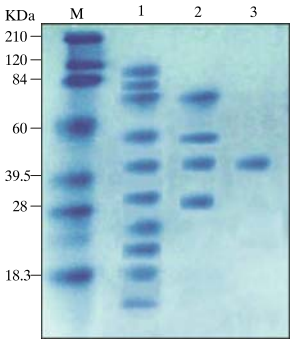


Fig. 3. SDS-PAGE profile of *A. flavipes* L-methioninase. Lanes: M, Marker; 1, Crude enzyme preparation; 2, DEAE-Cellulose; 3, Sephadex G_{100}.

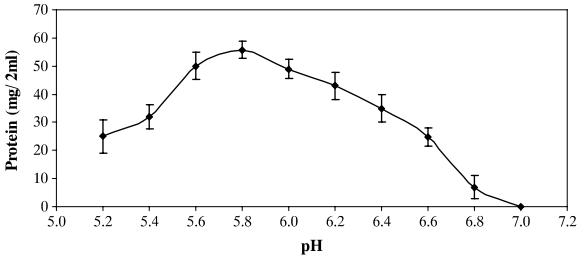


Fig. 4. Estimation of isoelectric point of L-methioninase, using different pHs of potassium phosphate buffer (5.2-7.0). The precipitated protein was quantified.

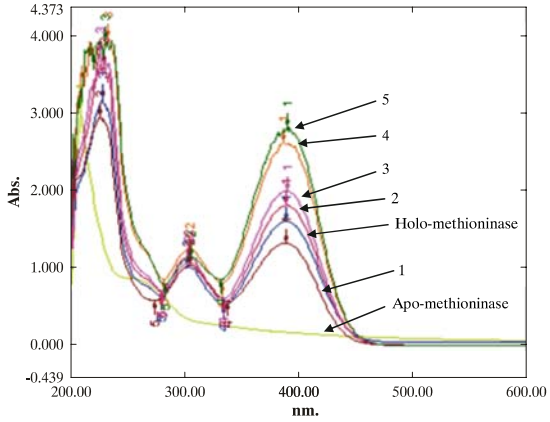


Fig. 5. Absorption spectra of holo and apo-methioninase from *A. flavipes.* Apo-methioninase obtained by preincubation of the holo-enzyme (2.2 mg/ml) in potassium phosphate buffer (pH 7.8) with 10 mM hydroxylamine. Reconstruction of the holo-enzyme by incubation of the apo-methioninase with 0.04 mM PLP (1), 0.08 mM PLP (2), 0.1 mM PLP (3), 0.15 mM PLP (4) and 0.2 mM PLP (5).

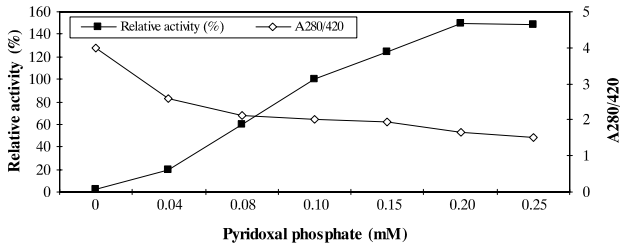


Fig. 6. Dissociation and reconstitution of the holo-methioninase of *A. flavipes.* Apo-L-methioninase was incubated with various concentrations of PLP, then measuring the spectral and activity profiles.

420, which ensured the protein concentration and pyridoxal phosphate linkage. Notably, the decreasing on the ratio of A280/420 due to the gradual addition of pyridoxal phosphate was parallel to restoring enzyme activity (Fig. 6). Unlike the nil activity of apoenzyme with A280/420 4.01, the enzyme completely retained its catalytic structural form upon addition of 0.15 mM pyridoxal phosphate (A280/420 1.7), with about 1.5-fold activity increase compared to the native holo-methioninase (A280/420 2.6).

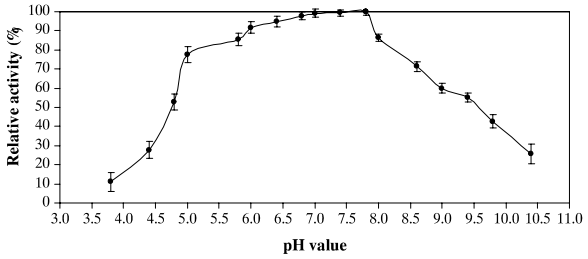


Fig. 7. pH stability profile of *A. flavipes* L-methioninase. The enzyme was preincubated for 2 h at various pHs (3.8-10.4), then measuring the residual demethiolating activity.

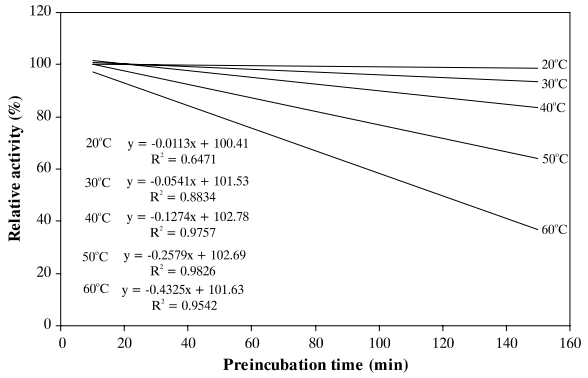


Fig. 8. Thermal stability profile of *A. flavipes* L-methioninase. After incubation of the free enzyme in different temperature (20-60°C) at various periods (30-150 min), the residual activity was determined by the standard assay method.

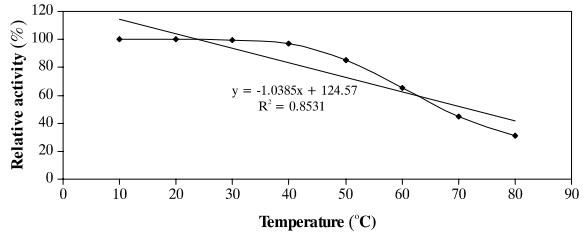


Fig. 9. Thermal inactivation profile. *Tm* is temperature degree at which the enzyme retains half of its initial activity at 60 min.

Determination of the L-methioninase glycosyl-residues

Structural glycosyl-units of *A. flavipes* L-methioninase were not detected. The absence of glycosyl residues from the purified fungal enzyme indicated a lack of glycosylation for this enzyme

Biochemical properties of L-methioninase

Optimum pH and pH stability: The optimum pH for L-methioninase activity was 7.8 using L-methionine as a substrate (data not shown). At pH 3.0, the enzyme lost about 73.8% of its initial activity. Also, at pH 10.6, only 23.9% of the enzyme activity was retained, compared to the optimum pH. The rate of enzyme inactivation was higher at more acidic

and alkaline pHs compared to the optimum pH.

The pH stability of the enzyme was assayed by preincubating the enzyme without substrate in a pH range (3.8-10.4) for 2 h. From the pH stability curve (Fig. 7), the enzyme was maximally stable at pHs ranging from 6.0 to 8.0. The rate of enzyme denaturation was significant after incubating the enzyme for 2 h at pH 3.4 and 10.4, retained about 11.1 and 25.8%, respectively, of its initial activity. Apparently, *A. flavipes* L-methioninase was catalytically stable in a neutral to slightly alkaline pH range.

Optimum temperature and thermal stability: The optimum temperature for L-methioninase activity was determined using a reaction mixture of pH 7.8, incubated at various temperatures. The maximum enzyme activity (33.1 U/ml) was observed at 35°C followed by a gradual decrease until it retained only 48.4% of its activity at 60°C (data not shown). Furthermore, the activity of L-methioninase was reduced by about 44.1%, at 20°C compared to optimum.

The thermal stability of the enzyme was evaluated by preincubating the enzyme without substrate at different temperatures (20-60°C) for different times (30-150 min). From the heat stability profile (Fig. 8), the enzyme appeared catalytically stable below 40°C. The conformational structure of the enzyme was not affected by preincubating it at 20°C for 150 min or at 30°C for 60 min. Unlike the relative structural stability of L-methioninase at 20°C, the rate of enzyme inactivation increased with higher temperatures and exposure times. The higher the temperature, the higher the denaturation rate of the enzyme as reflected in the half-life times at 20, 30, 40, 50, and 60°C which were 74.3, 15.8, 6.9, 3.4, and 1.9 h, respectively. The enzyme retained 62.9 and 36.6% of its activity by preheating at 50°C and 60°C, respectively, for 150 min. At 40°C, the enzyme lost about 9% of its activity by preheating for 90 min. From the thermal inactivation profile (Fig. 9), the *Tm* value of purified L-methioninase was 71.8°C using potassium phosphate buffer (pH 7.8).

Inhibitors of L-methioninase

The effects of various inhibitors and activators on the activity of *A. flavipes* L-methioninase are summarized in Table 2. L-Methioninase was significantly inhibited by DL-propargylglycine and hydroxylamine (10 mM), retaining only about 1.9 and 2.2% of its initial activity. The activity of L-methioninase was apparently not affected by EDTA, chelating agent, or Zn^{2+} ions. The activity of L-methioninase was significantly inhibited by about 4-fold by adding PMSF (1 mM), 2-mercaptoethanol, Cu^{2+}, and Fe^{2+} (10 mM). Guanidine-HCl, SDS, Hg^{2+}, and DTT had an inhibitory effect on enzyme activity by 83.8, 87.5, 56.5, and 40.9%, respectively. Triton X-100 had a significant denaturing effect on enzyme activity compared to Tween 80, as reflected by the relative enzyme activities (46.6 and 80.5%, respectively). The residual activity of L-methioninase in the presence of Mn^{2+}, Ca^{2+}, Se^{2+}, Cd^{2+}, and Li^{2+} was 58.4, 51.6, 69.9, 80.2, and 79.6%, respectively. Nevertheless, DMSO, K^+, and Na^+ had a similar and slightly inhibitory effect on enzyme activity by about 13.4, 14.9 and 10.2%, respectively. In contrast, enzyme activity was not affected by Co^{2+} and Ni^{2+}. Additionally, glucose, fructose, and potassium oxalate had no inhibitory effect on enzyme activity.

Table 2. Effect of inhibitors on *A. flavipes* L-methioninase activity

Compound	Conc. (mM)	Relative demethiolating activity (%)
Control	-	100
DL-propargylglycine	10	1.9±0.8
Hydroxylamine	1	3.8±0.3
	10	2.2±0.1
PMSF	1	25.2±3.9
Dithiothritol	10	59.1±2.5
Guanidine.HCl	10	16.2±1.0
SDS	10	12.5±1.8
2-Mercaptoethanol	10	23.3±0.8
Triton X-100	1	46.6±3.2
Tween 80	1	80.5±1.9
DMSO	10	86.6±1.8
EDTA	10	100.1±0.1
Hg^{2+}	10	43.5±2.5
Mn^{2+}	10	58.4±3.3
Zn^{2+}	10	97.1±1.2
Cu^{2+}	10	22.7±4.5
Ca^{2+}	10	51.6±3.2
Co^{2+}	10	109.1±1.2
Se^{2+}	10	69.9±2.4
Ni^{2+}	10	100.9±2.3
Cd^{2+}	10	80.2±1.2
Fe^{2+}	10	25.2±3.7
Li^{2+}	10	79.6±2.9
KCl	10	66.2±2.4
Na^+	10	89.8±1.3
Glucose	20	99.7±0.5
Fructose	20	96.7±2.1
Pot. oxalate	20	91.7±3.8

Substrate specificity of *A. flavipes* L-methioninase

The affinity of *A. flavipes* L-methioninase towards various substrates was evaluated. From the substrate specificity profile (Table 3), the enzyme had relative deaminating and demethiolating activities against various amino acids compared to L-methionine as a standard substrate. L-Methionine (100%) had an affinity to demethiolate DL-homocysteine (90%) to α-ketobutyrate, ammonia, H_2S, and cysteine (81.6%) to pyruvate, H_2S, ammonia, in addition to cystine (76.3%) to two cysteine units. *A. flavipes* L-methioninase had relative deaminating activity (52.1%) towards L-asparagine and deiminating activity of L-arginine (40.2%) to citrulline and ammonia compared to L-methionine as a standard substrate. Furthermore, the enzyme had a slight affinity to hydrolyze L-aspartic, L-glutamic acid, L-lysine, and D-glycine by about 15-35%. Otherwise, the enzyme had no activity to hydrolyze D-valine, D-alanine, L-phenylalanine, or *N*-acetylglucosamine. Furthermore, the enzyme displayed a relative proteolytic activity for casein by about 22.3%, as expressed by the amount of free amino acid.

Kinetic studies

DL-homocysteine, L-cysteine, and L-cystine displayed a higher affinity to be hydrolyzed by L-methioninase and were selected to be evaluated kinetically, compared to L-methionine as a

Table 3. Substrate specificity of *A. flavipes* L-methioninase

Substrate	Relative demethiolating activity (%)	Relative deaminating activity	
L-Methionine	100		
DL-homocysteine	90±3.5		
L-Cysteine	81.6±1.8		
L-Cystine	76.3±2.9		
L-Asparagine		52.1±3.7	
L-Aspartic acid		25.5±2.2	
L-Glutamine		32.1±1.5	
L-Glutamic acid		23.9±5.5	
L-Lysine		12.2±6.2	
L-Tyrosine[a]			3.4±1.3
L-Arginine		40.2±5.1	
D-Valine		0	
L-Alanine		0	
D-Glycine		32.7±3.8	
L-Phenylalanine[b]			0
N-Acetylglucosamine[c]			0
Urea		21.1±3.2	
Casein[d]			22.3±2.8

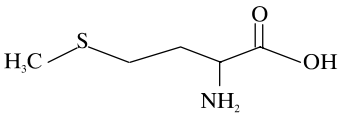
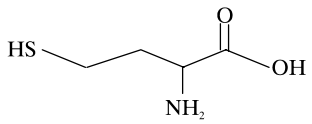
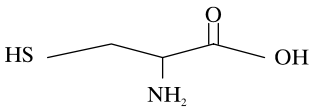
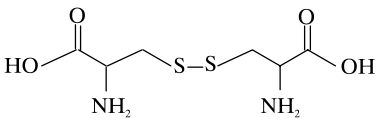
The reaction mixture contains 50 mM of each substrate in potassium phosphate buffer (pH 7.8) and 32.2 U/ml of L-methioninase.
[a] Tyrosine hydrolyzing activity was assayed by the method of Krishnaveni *et al.* (2009).
[b] L-phenylalanine lyase activity was checked as adopted by Fritz *et al.* (1976).
[c] *N*-Acetylglucosamine deacetylation was determined by method of Ride and Drysdale (1972)
[d] The proteolytic activity was assayed by the method of Ledoux and Lamy (1986).

substrate. The kinetic parameters Km, Vmax, and Kcat were determined for the enzyme using various concentrations of each substrate under standard assay conditions (data not shown). *A. flavipes* L-methioninase had maximum affinity to demethiolate L-methionine followed by DL-homocysteine as reflected by the Km values of 6.5 and 12 mM, respectively. Also, the highest catalytic efficiency of the enzyme was observed for L-methionine (14.1 S^{-1}) followed by homocysteine (9.3 S^{-1}). The enzyme displayed lower affinity to the disulfide dimmer L-cystine (Km, 38 mM and Vmax, 6.9 U/mg/min) compared to L-cysteine (Km, 17 mM and Vmax, 19.3 U/mg/min) (Table 4).

Cytotoxicity of *A. flavipes* L-methioninase

The cytotoxic effect of purified *A. flavipes* L-methioninase was explored based on hemoglobin levels and ALT and AST

Table 4. Kinetics of L-methioninase for different substrates

Substrate	Sterio-structure	End-productassay	Vmax (U/mg/min)	Km (mM)	Kcat (S^{-1})
L-Methionine		Methanethiol (CH_3SH)	39.6	6.5	14.1
L-Homocysteine		Hydrogen sulfide (H_2S)	20.7	12	9.3
L-Cysteine		Hydrogen Sulfide (H_2S)	19.3	17	7.8
L-Cystine		Thiol compounds	6.9	38	5.1

The kinetic parameters were determined by incubation of the enzyme (32.2 U/ml) in potassium phosphate buffer (pH 7.8) with various concentrations of each substrate (10-100 mM) under the standard assay conditions, then measuring the demethiolating activity of the enzyme. Maximum velocity (Vmax) was expressed by activity of enzyme in μmol of thiol compounds formed per minute per mg protein enzyme. Km is the substrate concentration (mM) at half of maximum velocity. Kcat is the maximum velocity of the enzyme per mol per sec.

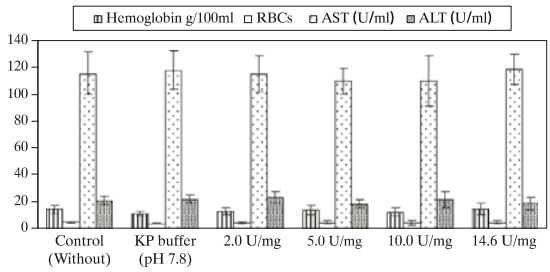


Fig. 10. Biochemical profile of mice groups injected with different doses of *A. flavipes* L-methioninase. The amount of hemoglobin, number of red blood cells, activities of AST and ALT were determined.

activities in mice injected with different doses of the enzyme preparation. As a result (Fig. 10), the amount of hemoglobin, number of red blood cells, and ALT and AST activities were apparently not affected by the enzyme preparation compared to control mice injected with potassium phosphate buffer (pH 7.8) or those left untreated. Additionally, no mice deaths were recorded even at high doses of L-methioninase (14.6 U/mg protein).

For the platelet aggregation assay, 50 µl of the enzyme (14.6 U/mg protein) was pipetted into human platelet-rich plasma (350 µl) and incubated for 20 min at room temperature. *A. flavipes* L-methioninase had no appreciable effect on inducing platelet aggregation, parallel to controls of normal platelet-rich plasma. Moreover, the hemolytic activity of the purified *A. flavipes* L-methioninase was evaluated using a blood agar assay (Fig. 11). The enzyme displayed no hemolytic activity to human blood.

Discussion

L-Methioninase is a common anticancer agent to various types of tumor cell lines (Mecham *et al*., 1983; Hoffman, 1984). The therapeutic response of bacterial L-methioninase is usually associated with multiple immunogenic reactions (Yang *et al*., 2004); thus, searching for a new enzyme with unique therapeutic properties is a challenge for many biotechnology laboratories. The current study was conducted to purify and characterize L-methioninase from *A. flavipes* solid cultures using chicken feathers as a substrate to maximally exploit the enzyme from academic and practical views. L-Methioninase was purified as an extracellular enzyme using anion-exchange and gel-filtration chromatography. Following the chromatography, the enzyme specific activity increased by 12.1 fold with a 39.8% yield. A successive set of purification steps was required to achieve a higher purification fold from the solid state fermentation cultures. The existence of multiple proteolytic and keratinolytic enzymes in the culture filtrate of feather growing microbes was detected for several bacterial species such as *Streptomyces albidoflavus* and *Kytococcus sedentarius* (Bressollier *et al*., 1999; Longshaw *et al*., 2002). The solid cultures of feather-grown *Microsporum gypsum* (Kunert, 1992), *Trichophyton rubrum* (Monod *et al*., 2005), and *Chrysobacterium* sp. (Riffel *et al*., 2007) contain disulfide reductases that synergize with proteases to hydrolyze feathers. Proteases were observed in the crude and partially purified L-methioninase from the feather-grown *A. flavipes* (El-Sayed, 2009b). Consistently, similar protocols have been reported for purifying L-methioninase from bacterial species (Tanaka *et al*., 1976; Nakayama *et al*., 1984; Lishko *et al*., 1993).

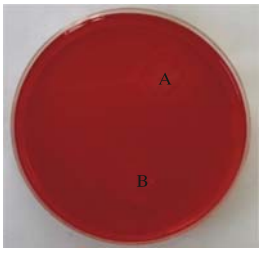


Fig. 11. Haemolytic activity of the purified *A. flavipes* L-methioninase. Enzyme activity 10 U/mg protein (A) and 14.6 U/mg protein (B).

The purified L-methioninase appeared as a single band on the denaturing gel. The molecular weight of the purified enzyme under denaturing conditions was 47 kDa, with four mass-identical subunits (Fig. 3). The appearance of a single band on SDS-PAGE after the third chromatographic step ensured the efficiency of the current purification system to purify L-methioninase from the cultures of feather-grown *A. flavipes*. Coincident with our results, the molecular weight of L-methioninase from bacterial (Nakayama *et al*., 1984; Dias and Weimer, 1998; Takakura *et al*., 2006) and protozoal species (Tokoro *et al*., 2003) was in the range of 44 to 48 kDa. Nevertheless, L-methioninase from *C. sporogenes* contains two pairs of non-identical subunits (Kreis and Hession, 1973). The pI for the purified *A. flavipes* L-methioninase was close to 5.8, which was consistent with that reported for the enzyme from *P. putida* (Ito *et al*., 1976). The pIs of L-methioninase from *C. sporogenes* (Kreis and Hession, 1973) and *E. histolytica* (Tokoro *et al*., 2003) are 4.2 and 6.1, respectively. Structurally, the amino acid profile (basic/acidic ratio) of *A. flavipes* L-methioninase is closer to a pseudomonad enzyme than a clostridial one. Therapeutically, the pI of fungal L-methioninase is far from normal blood pH (7.4), which increases the potential of this enzyme for tumor therapy.

The purified *A. flavipes* L-methioninase displayed two absorption maxima at 280 nm, indicating a protein containing aromatic amino acids, and at 420 nm due to the aldimine linkage of the pyridoxal phosphate aldehyde group and the lysine amino group on the PLP-enzyme binding domain. The absorption spectra of *A. flavipes* L-methioninase was typical of other pyridoxal-dependent enzymes (Dias and Weimer, 1998; Bertoldi *et al*., 2002; Saha *et al*., 2009). After incubating the enzyme with 10 mM hydroxylamine, the apo-methioninase produced only one peak at 280 nm, and the 420 nm peak disappeared due to the dissociation of the pyridoxal phosphate from the holo-enzyme. Similar results were observed for the *P. ovalis* enzyme (Tanaka *et al*., 1976).

The reconstitution of the holo *A. flavipes* L-methioninase was monitored structurally and catalytically by incubating apo-methioninase with different levels of pyridoxal phosphate as revealed by the ratio of A280/420. The ratio of A280/420 is a relevant indicator of the structural reconstitution of pyridoxal

phosphate-dependent enzymes (Johnston *et al*., 1979; Zhu *et al*., 2008). The ratio of A280/420 decreased from 4.01 to 1.7, upon addition of pyridoxal phosphate (0.2 mM) to the apo-enzyme, indicating reconstitution of the fully active holo-enzyme form. Consistently, the A280/420 for the freshly purified *Pseudomonas ovalis* L-methioninase is 3.90 followed by an increase to 4.7, due to the dissociation of pyridoxal phosphate upon repeated freezing and thawing (Johnston *et al*., 1979). Unlike *P. ovalis* L-methioninase, which lacks the ability to restore its original activity by dialysis against pyridoxal phosphate (Johnston *et al*., 1979), the *Trichomonas vaginalis* enzyme (Lockwood and Coombs, 1991) restores more than 90% of its activity with the addition of 0.1 mM PLP, while the *A. flavipes* enzyme reconstituted to its fully structural catalytic state with the addition of pyridoxal phosphate (0.2 mM). Similar results were reported by Zhu *et al*. (2008) for the responsiveness of apo-cystathionine γ-lyase by preincubating with pyridoxal phosphate.

The glyco-proteinic nature of the enzyme was evaluated for the number of glycosyl units associated with the enzyme. The enzyme had no sugar moieties, suggesting post-translational modification of this enzyme by a method other than glycosylation. Glycosylation of *P. putida* L-methioninase is usually associated with higher immunogenic reactions (Lishko *et al*., 1993). Currently, there are no studies that have elucidated the mechanism of L-methioninase post-translational modifications from fungi.

A. flavipes L-methioninase is optimally active in neutral to slightly alkaline pH (7.8). The significant decrease in the enzyme activity at pH 4-6 may be attributed to the isoelectric point of the enzyme (pI, 5.8) and/or L-methionine precipitation. The optimum pH for enzyme activity coincided with that reported for bacterial (Nakayama *et al*., 1984; Manukhov *et al*., 2005) and fungal L-methioninase (Ruiz-Herrera and Starkey, 1969). The pH stability of the purified L-methioninase was observed maximally at pHs ranging from 7.0 to 8.0. Similar results were obtained for the enzyme from other bacterial sources (Tanaka *et al*., 1976; Nakayama *et al*., 1988; Dias and Weimer, 1998), suggesting a similar conformational tertiary structure of the *A. flavipes* L-methioninase. The lower stability of the enzyme at higher and lower pHs may be attributed to the dissociation of pyridoxal phosphate or unfolding of the enzyme active state. Practically, lower enzyme stability in acidic conditions may be due to the closeness to the isoelectric point (5.8). The pH stability range of our enzyme appears higher than the pH of blood (7.4), which may sustain the enzyme clinically.

Maximum L-methioninase activity was observed at 35°C, which was consistent with that reported for the enzyme from *P. putida* (Nakayama *et al*., 1984) and *Aspergillus* sp. (Ruiz-Herrera and Starkey, 1969). The optimum temperature for purifying L-methioninase was slightly higher than the partially purified enzyme (El-Sayed, 2009b).

The enzyme displayed a relative catalytic stability below a temperature of 40°C. The half-life times of the enzyme at 20, 30, 40, 50, and 60°C were 74.3, 15.8, 6.9, 3.4, and 1.9 h, respectively. Consistently, *Brevibacterium linens* L-methioninase had a thermal stability below 40°C (Dias and Weimer, 1998). The higher inactivation of the enzyme at higher temperature may be attributed to the thermal denaturation of the enzyme subunits.

A. flavipes L-methioninase was completely inhibited by DL-propargylglycine, a cysteine suicide inhibitor, which is similar to other PLP-dependent enzymes in the γ-subfamily (Johnston *et al*., 1979; Dias and Weimer, 1998; Zhu *et al*., 2008). The enzyme was also significantly inactivated by the carbonyl reagent, hydroxylamine, which may be ascribed to the dissociation of pyridoxal phosphate from holo-methioninase, typical of other PLP enzymes (Lockwood and Coombs 1991; Bertoldi *et al*., 2002; Martinez-Cuesta *et al*., 2006). EDTA had no inhibitory effect on enzyme activity, ensuring the non-metallic nature of this enzyme. Additionally, L-methioninase was strongly inhibited by PMSF, 2-mercaptoethanol, SDS, and DTT suggesting the presence of a cysteine/disulfide bond for maintaining the molecular catalytic folding state of the enzyme, consistent with the results obtained by Thong *et al*. (1987) and Lockwood and Coombs (1991). The inhibitory effect of guanidine-HCl on *A. flavipes* L-methioninase activity could be ascribed to the dissociation of pyridoxal and/or enzyme denaturation (Saha *et al*., 2009). Furthermore, Triton X-100 and Tween 80 inhibited enzyme activity through physical interference of the hydrophobic/hydrophilic amino acids. However, the enzyme activity was not affected by the presence of organic compound such as glucose, fructose, and potassium oxalate.

The *A. flavipes* enzyme had relative catalytic activity towards different amino acids (Table 3). The enzyme had a relative affinity to demethiolate DL-homocysteine, L-cysteine, and L-cystine. Additionally, the enzyme had slight deaminating activity towards various amino acids, assuming the hydrolytic ability to the C-N bonds. The multifunctional activity of L-methioninase for hydrolyzing the C-S and C-O rather than the C-C bonds is commonly observed for bacterial (Kreis and Hession, 1973; Tanaka *et al*., 1976; Dias and Weimer, 1998; Martinez-Cuesta *et al*., 2006), protozoal (Lockwood and Coombs, 1991; Sato *et al*., 2008), and fungal enzymes (Ruiz-Herrera and Starkey, 1969; El-Sayed, 2009b). In contrast, the enzyme had no activity to deacetylate *N*-acetyl-glucosamine, ensuring the non-glycolipolytic activity of this enzyme, which emphasizes a clinical application. The relative deaminating activity of *A. flavipes* L-methioninase for different amino acids, assumed the similarity in the spatial arrangement of the α-carbon of these amino acids. Moreover, the enzyme had relative proteolytic activity towards casein as a substrate. Catalytically, the dual-function of the enzyme for demethiolation and proteolysis might be attributed to gene overlapping, resulting in both processes. Similar assumptions have been proposed for histidinol dehydrogenase and imidazolyl acetol phosphate amino-transferase from *Salmonella typhimurium* (Yourno *et al*., 1970; Rechler and Bruni, 1971).

A. flavipes L-methioninase had a relative higher affinity towards DL-homocysteine, L-cysteine, and L-cystine than L-methionine. The relative activity towards the sulfur amino acids may be attributed to the similar molecular configuration of the α, β carbons (Table 4). The affinity of the enzyme for these amino acids was arranged descendingly; L-methionine, DL-homocysteine, L-cysteine, and L-cystine according to their Km and Kcat values. The activity of L-methioninase as a homocysteinase and cystalysin is an undesirable criteria that limits its clinical feasibility, because normal cells can utilize DL-ho-

mocysteine in the absence of L-methionine, for DNA methylation, polyamine, and glutathione synthesis (Cellarier *et al*., 2003), as reviewed by El-Sayed (2010). However, L-methioninase from *Brevibacterium linens* (Dias and Weimer, 1998), *Clostridium sporogenes* (Kreis and Hession, 1973), and *P. ovalis* (Tanaka *et al*., 1977) display more affinity to DL-homocysteine than L-methionine as revealed from the Km values. Otherwise, the enzyme from *Aspergillus* Rs-1 (Ruiz-Herrera and Starkey, 1969) and *E. histolytica* (Sato *et al*., 2008) has relative activity of 34% and 20% on DL-homocysteine, respectively, regarding L-methionine as a standard substrate, which ensured our results for the *A. flavipes* enzyme. Consequently, the kinetic limitations of bacterial enzymes that hinder their therapeutic use may be reduced by eukaryotic enzymes. Further studies on this enzyme are underway by our lab to minimize the affinity of this enzyme for the different amino acids and to maximally exploit this enzyme from a therapeutic and economical point of view.

The cytotoxic effect of the purified *A. flavipes* L-methioninase was evaluated *in vivo* using Swiss Albino mice. From the biochemical analysis, the enzyme had no stimulatory effect on ALT and AST activities, compared to controls injected with potassium phosphate buffer, revealing the absence of a direct effect on liver. ALT and AST are the most relevant indicators of potential of liver dysfunction (Pratt and Kaplan, 2000). Moreover, the enzyme had neither an appreciable effect on human platelet aggregation nor hemolytic activity.

In conclusion, this study highlights the potential for purifying and producing L-methioninase from *A. flavipes* using chicken feathers as a substrate for solid state fermentation. The *A. flavipes* enzyme was characterized by favored kinetic and catalytic properties over the currently applied bacterial enzymes, which are used as potent therapeutic agents. The spectroscopic analysis revealed the efficiency of this enzyme to restore its fully active state upon addition of PLP. In addition, the enzyme did not display toxicity signs as shown by the liver function enzymes activity, platelet aggregation, and hemolysis assays. To the best of our knowledge, this is the first report to purify and characterize this enzyme from a fungal source using solid state fermentation.

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