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Purification and Characterization of New Alkaline L-methioninase from *Aspergillus ustus* AUMC 1051 Grown under Solid-State Fermentation Conditions.

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ALKALINE L-methioninase (E.C.4.4.1.11) from *Aspergillus ustus* AUMC 1051 was obtained in a good yield amounting to 1321 Uml^{-1} (99.56 Ug^{-1} bran) under solid state fermentation (SSF) of wheat bran. The enzyme was purified 15.83-fold with 62.63% yield after three steps of purification involved ammonium sulfate precipitation, Sephadex G-100 gel filtration and DEAE-cellulose ion exchange chromatography. The purified enzyme had a molecular mass of 46 kDa under denaturating conditions and an isoelectric point of 6. Maximal activity was recorded at pH 8.5 and 35^{o}C. Good stability of the purified enzyme was detected over wide pH values ranging from 8 to 10 and temperature up to 50^{o}C. The enzyme retained its full activity after 6 days of storage at 4^{o}C. Four weeks was found the $T_{1/2}$ of its activity. V_{max} and $K_{m,}$ of the purified enzyme were found to be 820 Uml^{-1} and 1.6 mM, respectively. Alkaline L-methioninase activity was stimulated by Na^{+} and Co^{+2} and strongly inhibited by Hydroxylamine, iodoacetate, Hg^{+2} and Cu^{+2}. The enzyme was proved to be glycoprotein containing -SH group in its catalytic site.

Keywords: Solid-state fermentation, Alkaline L-methioninase, Purification, Characterization, *Aspergillus ustus*.

L-methioninase (E.C.4.4.1.11) is a pyridoxal L-phosphate dependent enzyme that catalyzes the deamination and demethiolation of L-methionine to α-ketobutyrate, methanethiol and ammonia (Ruiz-Herrera and Starkey, 1969).

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 and Yamamoto *et al.*, 2003).

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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 as far the authors are aware.

Material and Methods

Fungal strain and growth conditions

Aspergillus ustus AUMC 10151 was selected as the most potent fungus for production of L- alkaline methioninase from our preliminary studies (Abu-Tahon and Isaac, 2016). *A. ustus* was grown on the optimized solid state fermentation medium utilizing wheat bran as carbon and nitrogen sources. Maximum alkaline L-methioninase was achieved at initial moisture content of 71.5%, inoculum size of 2 mL of spore suspension, initial pH 8.5, incubation period 8 days at 30°C and supplementation of the salt basal medium with pyridoxine (100 µg/ml) and beet molasses (20%, v/v).

Extraction of alkaline L-methioninase

At the end of the incubation period, the content of each flask was thoroughly mixed with 40 ml of 0.2 M Glycine-NaOH buffer (pH 8.5) using a rotary shaker at 200 rpm for 30 min. The whole content of each flask was filtered through muslin cloth and the pooled filtrate was centrifuged at 4000 rpm for 15 min. at 4.0 ^{o}C. The clear supernatant was collected as the crude enzyme preparation.

Materials

Nessler's reagent, Folin reagent, pyridoxine, Sephadex G-100 and diethyl amino ethyl cellulose (DEAE–cellulose) were obtained from Sigma-Aldrich (St. Louis, Mo).

L-methioninase assay

The activity of alkaline L-methioninase was determined using Nessler's reagent (Thomposon and Morrison, 1951) with some modification as follow: 1ml of the enzyme preparation was incubated with 1ml of 1% L-methionine in 0.2 M Glycine-NaOH buffer (pH 8.5) and 0.1 ml of pyridoxal phosphate, for 1 hr at 30 ^{o}C. The enzyme activity was stopped by adding 0.5 ml of 1.5 N trichloroacetic acid, followed by centrifugation at 5000 rpm for 5 min. The released ammonia was determined using 0.5 ml Nessler's reagent; the developed color was measured at 480 nm. Enzyme and substrate blanks were used. One unit of L-methioninase was defined as the amount of enzyme that liberates one µmole of ammonia/h under optimal assay conditions. Specific activity of L-methioninase was expressed as the activity of enzyme in term of units per milligram of protein.

Protein Determination

The method used was described by Lowry *et al.* (1951) using bovine serum albumin as the standard.

Enzyme purification

Enzyme purification was started with dialysing 200 ml of crude enzyme preparation (CEP) of *A. ustus* overnight against 0.2 M Glycine-NaOH buffer (pH 8.5) at 4^{o}C. The enzyme was precipitated from the cell-free dialysate (CFD) by gradual addition of $(NH_4)_2SO_4$ using the range of saturation from 50 to 90%. The precipitate was collected by centrifugation, dissolved in minimal of the same buffer, applied to a Sephadex G-100 gel filtration column (1.5×4.5 cm) and eluted with the same buffer. The fractions possessing the highest L-methioninase activity were collected, pooled, concentrated and applied to ion-exchange chromatography column containing diethyl amino ethyl cellulose (DEAE–cellulose). Elution in this case was achieved by a linear gradient of NaCl up to 0.5 M. The fractions containing the highest activity of the purified enzyme were collected, desalted by dialysis, lyophilized by Labconco Freeze Dryer at -65^{o}C and 250 µ bars and stored at 0°C for further investigations.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

SDS-PAGE was carried out according to Laemmli (1970), using 10% polyacrylamide. Proteins were detected by Coomassie Brilliant Blue R250 staining.

The isoelectric point for 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 (5.0-7.0) using potassium phosphate buffer at 4°C. After 12 hr of incubation, the enzyme was precipitated by centrifugation at 10,000 rpm for 10 min. and 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.

Determination of kinetic parameters

The optimum pH of the enzyme activity was investigated by measurements at 30 ^{o}C in different buffers covering the pH range of 6-11. These buffer solutions were 0.1 M Citrate-phosphate, pH 6.0-8.0 and 0.2 M Glycine-NaOH, pH 8.5-11.0. The pH stability of the enzyme was determined by measuring its residual activity after incubation for different periods in the buffers with different pH values. Optimum temperature of the enzyme activity was determined by incubating enzyme at various temperatures ranging from 20 to 60^{o}C in 0.2 M Glycine-NaOH (pH 8.5). Thermostability of the enzyme was measured after incubating the enzyme at different temperatures for different periods in the same buffer. Enzyme was incubated with various concentrations of L-methionine in 0.2 M Glycine-NaOH (pH 8.5) at 35°C and different kinetic parameters were calculated. Michaelis constant (Km) and maximum velocity

(V_{max}) of enzyme activity were calculated by linear regression from Lineweaver-Burk plot (Lineweaver and Burk, 1934). Effect of various metal ions and inhibitors on activity of the purified alkaline L-methioninase was assessed by following the standard assay conditions at 1 and 10 mM final concentration.

Substrate Specificity of Purified L-Methioninase.

This experiment was designed to evaluate the specificity of the purified alkaline L-methioninase towards various substrates, namely, L-methionine, L-aspargine, L- aspartic acid, L-glutamine, L-glutamic acid , L-lysine and urea. Enzyme activity was assessed by the nesslerization method as described above.

Statistical Analysis

The obtained data were statistically analyzed with SPSS (Scientific Package for Scientific Social Studies, version 20), in which the equations of the hypothesis tests, including the mean, standard deviation, T-statistics value and probabilities (p) were used.

Results and Discussion

Purification

A summary of purification procedure for the produced enzyme was presented in Table 1. The results reveal that dialysis of the CEP resulted in reduction of the enzyme activity which can be attributed to the loss of certain metallic ions as stated by Ghareib and Abu-Tahon (2013). Precipitation of proteins from the CFD with 70% saturation of ammonium sulfate provided a fraction containing the highest enzyme preparation. The same precipitating agent was previously found convenient for maximal precipitation of L-methioninase from *A. flavipes* (El-Sayed, 2009 and 2011). Completion of enzyme purification was achieved by gel filtration through SephadexG-100 and ion-exchange chromatography with DEAE-cellulose. The present study showed that *A. ustus* has produced L-methioninase with purification of 15.83-fold and yield of 62.63% which is better compared to *A. flavipes* which has shown 12.1 fold purity with 39.8% yield (El-Sayed, 2011).

TABLE 1. Purification profile of L-methioninase of *Aspergillus ustus* AUMC 1051 .

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Yield (%)	Purification fold
CEP (200 ml)	264200	2654	99.56	100	1.0
CFD	242973	2654	91.55	91.97	0.91
Ammonium sulphate (70%)	220350	1180	186.73	83.40	1.90
Sephadex G-100	190645	550	346.62	72.15	3.50
DEAE-cellulose	165479	105	1575.99	62.631	15.83

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Figure 1 shows the electrophoretogram of the purified alkaline L-methioninase from *A. ustus* as determined using SDS-PAGE. The molecular weight of the purified enzyme was estimated after final stage of purification to be 46 kDa. The appearance of L-methionine as a single band ensures the homogeneity and purity of the enzyme. Coincident with our results, the molecular weight of the purified L-methioninase produced by various bacterial (Nakayama *et al*., 1984; Dias and Weimer, 1998 and Takakura *et al*., 2006), protozoal (Tokoro *et al*., 2003) and fungal species (El-Sayed, 2009 and Selim *et al*., 2015) was in the range of 44 to 48 kDa.

The isoelectric point for L-methioninase

The *pI* for the purified *A. ustus* L-methioninase was close to 6, which is consistent with that reported for the enzyme from *E. histolytica* (Tokoro *et al*., 2003). Meanwhile, the *pI*s of L- methioninase from *C. sporogenes* (Kreis and Hession, 1973) and *A.flavipes* (El-Sayed, 2011) were found to be 4.2 and 5.8, respectively. Having the *pI* of the purified enzyme far from the normal blood pH (*i.e.* 7.4) may suggest a possible application in tumor therapy.

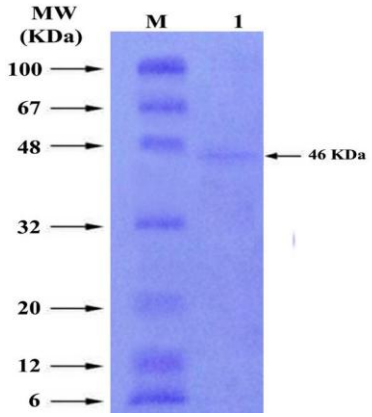

Fig. 1. SDS-PAGE analysis of the purified L-methioninase by *A. ustus* AUMC 1051. Lane 1, L- methioninase sample. Lane M, molecular weight standards (Marker).

Effect of pH on enzyme activity and stability

The results (Fig. 2) reveal that the purified enzyme showed a great activity at pH 8-9.5 with the maximum at pH 8.5. These results are in agreement with the previous findings on other L-methioninase from *Clostridium sporogenes* (Kreis and Hession, 1973), *Cladosporium cladosporioides* (Abu-Seidah and Youssef, 2000) and *A. flavipes* (El-Sayed, 2009). While, our results are in disagreement with that obtained from *Candida tropicalis* (Selim *et al.,* 2015).

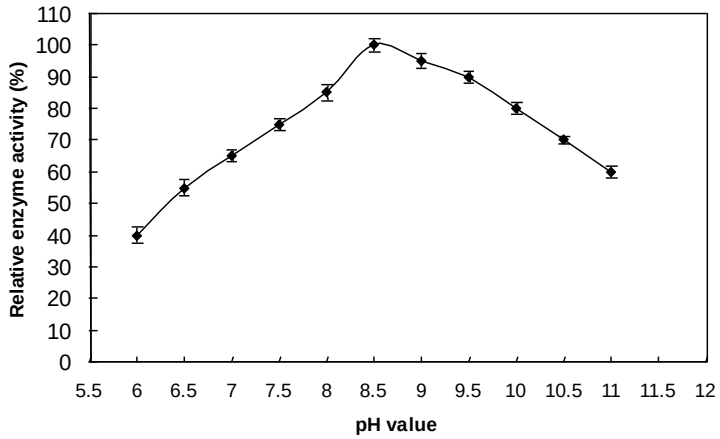

Fig. 2. Effect of pH value on activity of the purified alkaline L-methioninase of *A. ustus*.

The enzyme had a good stability on the alkaline side. It retained full activity after 30 min of incubation at pH 8.5 and about 85% of the original activity was restored at pH 9 (Fig. 3). After an hour at pH 8.5 90% of the original activity was recorded. Similar results are obtained by Ruiz-Herrera and Starkey (1969) and El- Sayed (2009).

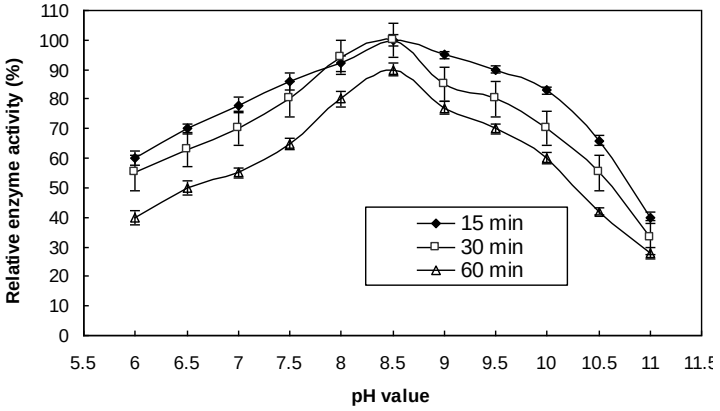

Fig. 3. Effect of pH value on stability of the purified alkaline L-methioninase of *A. ustus*.

Effect of temperature on enzyme activity and stability

Optimum activity of *A. ustus* alkaline L-methioninase in its purified state was recorded at 35°C (Fig. 4). At higher temperatures, a gradual decrease in enzyme activity was observed. This outcome may be explained by the fact that the temperature increases the reaction velocity and also affects the rate of enzyme destruction, producing a gradual fall in the concentration of active enzyme. Our

results were in complete accordance with that recorded for the enzyme of *Aspergillus* sp. by Ruiz-Herrera and Starkey (1969) and El- Sayed (2009). Other temperature optima were recorded for purified L-methioninase from other organisms. These were 30°C for the enzyme from *C. cladosporioides* (Abu-Seidah and Youssef, 2000) and *Citrobacter freundii* (Manukhov *et al*., 2005) and 37°C for that from *Entamoeba histolytica* (Sato *et al*., 2008). The inactivation of the enzyme at higher temperature may be attributed to the thermal denaturation of the enzyme subunits (Dias and Weimer, 1998).

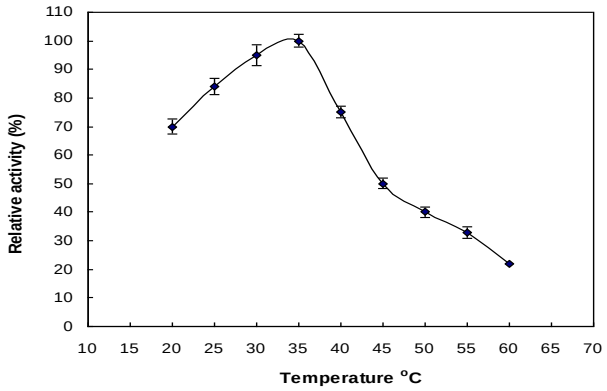

Fig. 4. Effect of temperature on activity of the purified alkaline L-methioninase of *A. ustus*.

The thermostability of the purified enzyme (Fig. 5) was proved as it could tolerate up to 45°C for an hour without loss of more than 25% of its initial activity. It also retained about 55% of this activity at 50°C and 35% at 60°C after heating for complete hour. These results reveal that this enzyme from *A. ustus* was less thermostable than those of *Candida tropicalis* (Selim *et al*., 2015) but more stable than that of *A. flavipes* (El-Sayed, 2011).

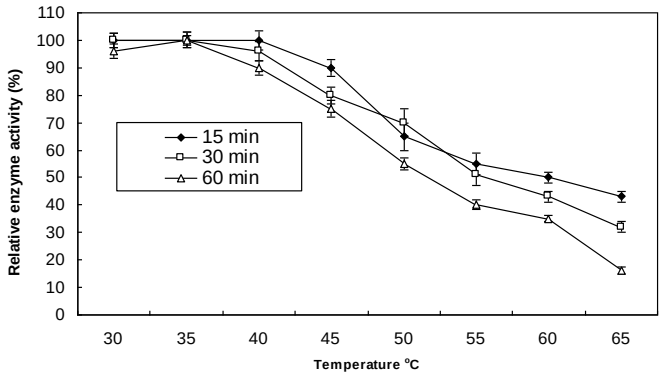

Fig. 5. Effect of temperature on stability of the purified alkaline L-methioninase of *A. ustus* .

The purified enzyme preparation was found stable for six days when stored at 4°C. T ½ of enzyme activity was found to be four weeks.

Substrate Specificity of Purified L-Methioninase

Substrate specificity of *A. ustus* L-methioninase revealed that the enzyme had a relative activity towards various amino acids. In Fig. 6 It was found that the enzyme showed the highest affinity towards L-methionine as a standard substrate. The enzyme activities towards L-asparagine and L-glutamine were 58% and 50%, respectively. While the enzyme activity towards the other substrates were less than 20%. Our results are agreed with EL-Sayed (2011) who reported that the L-methioninase purified from *A. flavipes* had relative catalytic activity towards the same investigated amino acids.

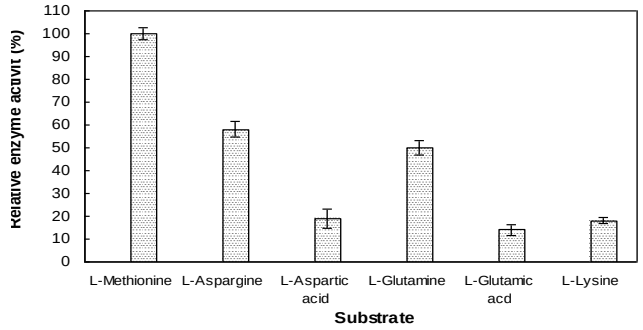

Fig. 6. **Substrate specificity of purified L-methioninase determination of kinetic parameters.**

Catalytic properties

V_{max} of purified enzyme was calculated to be 820 Uml^{-1} (Fig. 7). Km value was calculated from the Lineweaver-Burk plot of reciprocals of initial velocities and substrate concentrations to be 1.6 mmol (Fig. 8). This value is very near from that recorded for the same enzyme from *pseudomonas putida* (Takakura *et al*., 2004).

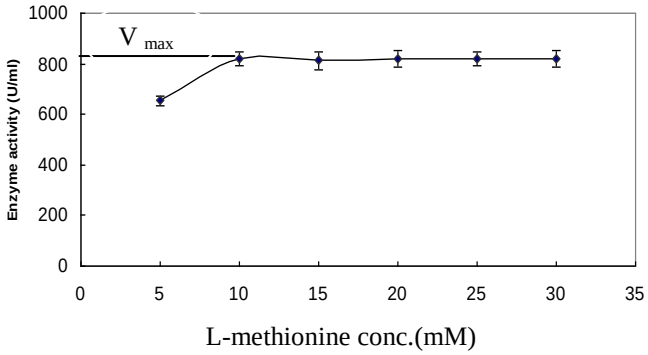

Fig. 7. **Effect of L-methionine concentration on activity of the purified alkaline L-methioninase of *A. ustus.***

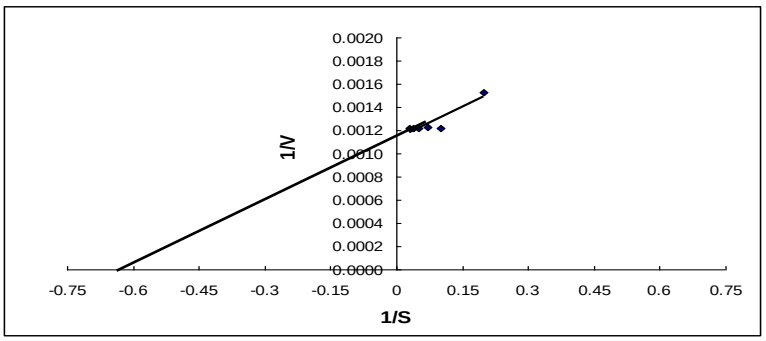

Fig. 8. **Lineweaver-Burk plot of the reciprocal of initial velocities and L-methionine concentrations.**

Effect of Different Metal Ions and Inhibitors on L-Methioninase activity

Metal ions may serve as activators or inhibitors in numerous enzymatically catalyzed reactions. Therefore, the effect of some metal ions on alkaline L-methioninase activity was investigated (Table 2). The results showed that Na^{+} and Co^{+2} act as potent activators, where the enzyme activity was significantly of the enzyme from autoproteolysis and thermal denaturation (Secades increased to 120 and 115%, respectively, of the original activity at a final concentration of 10 mM. Moreover, Ca^{+2}, $Mg^{+2}$and Ni^{+2} had slightly stimulatory effect on enzyme activity. On the other hand, the enzyme activity was inhibited in the presence of Cu^{+2}, Zn^{+2}, Hg^{+2} and Fe^{+2}. These results are in consistent with that reported for fungal L-methioninase (Abu-Seidah and Youssef, 2000, El-Sayed, 2009 and Selim *et al*., 2015). Generally, the stimulatory effect of metal ions may be ascribed to their stabilizing effect to the conformational structure, protection and Guijarro, 2001).

Presence of all enzyme inhibitors affected unfavorably its activity. The largest inactivation was recorded in presence of carbonyl reagents such as Hydroxylamine which is strongly inhibitor to the pyridoxal 5-phosphate insuring the pyridoxal dependence of this enzyme (Dias and Weimer, 1998). The enzyme was completely inactivated by a thiol reducing agent such as iodoacetate which react with sulfur amino acids (Lockwood and Coomps, 1991). The complete inhibition of L-methioninase with this agent provides evidence for the presence of –SH group in the active sites of enzyme.

TAPLE 2. Effect of some chemical components (metal ions and enzyme inhibitors) on activity of the purified alkaline L-methioninase.

Components*	Conc. (mM)	Relative enzyme activity (%)
Control	0.0	100
Metal ions		
K^+	1	100
	10	96
Na^+	1	100
	10	120
Ca^{+2}	1	100
	10	104
Co^{+2}	1	105
	10	115
Cu^{+2}	1	82
	10	44
Mg^{+2}	1	100
	10	102
Zn^{+2}	1	95
	10	72
Hg^{+2}	1	82
	10	44
Fe^{+2}	1	80
	10	52
Fe3+Fe	1	100
Ni^{+2}	10	107
Enzyme inhibitors		
SDS	1	84
	10	60
Hydroxylamine	1	15
	10	5
EDTA	1	94
	10	86
PMSF	1	25
	10	55
Iodoacetate	1	44
	10	23

*The enzyme was pre-incubated with these components (metal ions were added as chlorides) for 15 minutes at the two indicated concentrations.

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تنقية وتوصيف إنزيم إل- ميثيونينيز القلوى الجديد فى مزارع الحالة الصلبة لفطر اسبرجلس استس AUMC 1051

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ترجع أهمية إنزيم إل- ميثيونينيز كونه أحد الإنزيمات المستخدمة فى علاج الأورام السرطانية. وقد تم تنقية انزيم أل-ميثيونينيز القلوى المنتج من فطر اسبرجلس استسAUMC 1051 النامي على ردة القمح فى مزارع الحالة الصلبة حوالى ستة عشر مرة بانتاج يقدر ٦٢,٦٣% عن طريق الترسيب بكبريتات الأمونيوم، والترشيح الهلامى خلال أعمدة السيفادكس متبوعاً بالتبادل الأيونى فى أعمدة ثنائى ايثيل أمينوايثيل السيليلوز. وقدر الوزن الجزيئى للإنزيم المنقى بـ ٤٦ كيلو دالتون ونقطة التعادل الكهربى بـ ٦ . وقد سجل الإنزيم أقصى نشاط له عند الرقم الهيدروجينى ٨,٥ ، ودرجة حرارة ٣٥ درجة مئوية. كما سجل الانزيم ثباتاً جيداً عند الأرقام الهيدروجينية من ٨- ١٠ وعند درجات الحرارة حتى ٥٠ درجة مئوية. كما تبين احتفاظ الإنزيم بكامل نشاطه عند حفظه فى درجة حرارة ٤ مئوية وذلك لمدة ستة أيام فى الوقت الذى وصل النشاط الإنزيمى إلى النصف بعد أربعة أسابيع. وبتقدير النشاط الأقصى للإنزيم المنقى وثابت ميكائيل وجد أنهما يقدران بـ ٨٢٠ وحدة لكل مل و ١,٦ مللى مول. وقد وجد أن أيونات الصوديوم والكوبلت ينشطان النشاط الإنزيمى فى الوقت الذى يتم تثبيط نشاطه بقوة بأي من الهيدروكسيل أمين و خلات اليود وكذلك أيونات الزئبق والنحاس. وقد وجد أن الإنزيم يحتوى على مجموعة سلفهيدريل فى موقعه النشط.