POLISH JOURNAL OF FOOD AND NUTRITION SCIENCES

http://journal.pan.olsztyn.pl
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Pol. J. Food Nutr. Sci.
2009, Vol. 59, No. 3, pp. 211-217

GLUTAMINE AMIDOHYDROLASE FROM *PENICILLIUM POLITANS* NRC 510

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Key words: *Penicillium politans* glutaminase, fungal glutamine amidohydrolase, fungal glutaminase, glutaminase in filamentous fungi

Glutamine deamidating enzyme from *Penicillium politans* NRC510 catalyzed deamidation of glutamine to glutamic acid and ammonia. The enzyme was partially purified by a simple method of heating and Sephadex G-100 gel filtration. This procedure yields the partially purified enzyme with a 25% recovery of the activity in crude extracts. Specific activity of this partially purified enzyme is 133 U/mg. The partially purified enzyme hydrolyzes L-glutamine, D-glutamine L-asparagine and D-asparagine, while it cannot hydrolyze the other amide such as nicotinamide adenine dinucleotide, nicotinamide and acetamide under the same experimental conditions. The purified enzyme showed the maximal activity against L-glutamine at pH 7.5-8.5 and 60°C. The enzyme has a high salt tolerance, that shows high activity (75% of the original activity) in the presence of 15-30% NaCl. Exposure of the partially purified enzyme to 60°C for 30 min in the absence of the substrate, has no effect on its activity. While it was inhibited to a variable extent by addition of some substances such as $HgCl_2$, NaF, $CaCl_2$, $BaCl_2$ and $CuSO_4$ but was not affected by 2-merceptoethanol and iodoacetate. Product inhibition was recorded by addition of glutamic acid or NH_3 to the reaction mixture. Glutamic acid inhibition was a competitive type and the k_m and the k_i values were found to be 7.5 and 39.0 mol/L, respectively.

INTRODUCTION

Glutaminase (L-glutamine amidohydrolase EC 3.5.1.2) plays a catabolic role in the degradation of glutamine to carbon skeletons and also plays a role in maintaining the balance between glutamate and glutamine, the universal nitrogen donors of the cell. Glutamine is the major respiratory fuel and energy source of the rapidly proliferating tumor cells and that is why glutaminase has received much attention with respect to its therapeutic application for treatment of leukaemia [Roberts *et al.,* 1970] and thermostable glutaminase activity in six thermophilic strains of *Pseudomonas* has been reported by Rath & Subramanyam [1996].

It is well known that most of the basic flavor components of fermented condiments are amino acids produced by the enzymatic degradation of proteins contained in the raw materials and among them; L-glutamic acid is a widely acclaimed flavor-enhancing amino acid. For example, the unique flavor of fermented soy sauce is attributed mainly to glutamic acid [Ohshita *et al.,* 2000; Bhattacharya *et al*., 2001; Ito *et al*., 2002]. Glutaminase is ubiquitous in microorganisms including bacteria, yeast and fungi in general, glutaminases from *E. coli* [Prusiner *et al*., 1976], *Bacillus subtilis* [Yokotsuka *et al.,* 1987; Shimizu *et al.,* 1991]. In yeast, glutaminase from *Saccharomyces cerevisiae* and *Cryptococcus albidus* [Soberon & Gonzalez 1987; Ohshita *et al.,* 2000], and in fungi, such as *Rhizobium etli* [Duran *et al.,* 1996] and *Aspergillus oryzae* [Yano *et al.,* 1988; Thammarongtham *et al.,* 2001] glutaminase has been isolated and well studied. As far as the occurrence of glutaminase producers with respect to the environment is concerned, so far the majority of microorganisms identified to possess glutaminase activity were isolated from soil except for reports from aquatic (marine) environment [Moriguchi *et al.,* 1994; Dharmaraj *et al.,* 1977; Sabu *et al*., 2000].

The present research demonstrates the detection of L-glutamine and L-asparagine deamidating activities in extracts of a filamentous fungus and provides data indicating that one enzyme, in *Penicillium politans* NRC 510 extracts, is involved in deamidation of both amino acids.

MATERIALS AND METHODS

Chemicals

L-glutamine, L-asparagine, acetamide, L-glutamic acid and L-aspartic acid were products of BDH Chemicals Ltd. Nicotinamide adenine dinucleotide (NAD) and nicotinamide were products of Sigma. Sephadex G-100 was from Pharmacia Fine Chemicals.

Organism

The *Penicillium politans* NRC 510 was from the culture collection of the Department of Microbial Chemistry, National Research Centre, Cairo, Egypt.

Medium

The organisms were grown and kept on slants of solid modified Czapek Dox's medium containing (g/L tap water): glucose, 30; $Na\ NO_3$, 2.0; $KH_2\ PO_4$, 1.0; $Mg\ SO_4\ 7\ H_2O$, 0.5; KCl, 0.5 and agar 20.

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Preparation of fungal extracts

The 4 days old mats were grown on liquid modified Czapek-Dox's medium at 28°C. The cells were harvested by filtration, washed thoroughly with distilled water, blotted dry with absorbent paper then ground with cold washed sand in a chilled mortar and extracted with cold distilled water. The slurry so obtained was centrifuged at 1522 ×g for 10 min and the supernatant was used as the crude enzyme preparation.

Assay of L-glutaminase

An assay method has been reported where the rate of hydrolysis of glutaminase is determined by direct nesslerization of ammonia released according to Borek *et al.* [2004]. Protein of the extracts was estimated by the method of Ohnistti & Barr [1978], and that of the eluted fractions was determined by UV absorption according to the method of Jackson *et al.* [1981].

Fractionation of the extract proteins using Sephadex G-100 column chromatography

The crude extracts were heated at 60°C for 20 min, immediately cooled in ice, and then centrifuged at 4830 ×g for 10 min at –20°C. The supernatant (10 mL) was then loaded on a Sephadex G-100 column (1.5 x 40 cm) which was washed with 0.02 mol/L Tris-HCl buffer at pH 6. Elution of protein was then carried out by 0.02 mol/L Tris-HCl buffer at pH 6. Fractions of 5 mL each were collected at room temperature (25°C) at a flow rate of about 30 mL/h. At the end of the fractionation, the activity of each fraction was tested with L-glutamine as a substrate.

The assay reaction mixture of the eluted fractions contained (in 1 mL vol.): 0.4 mL of each fraction, 5.0 μmoles substrate and 80 μmoles Tris-HCl buffer at pH 8. The time of the reaction was 30 min and the temperature was 50°C.

Specific activity was expressed as μmoles of NH_3 released from 5.0 μmoles substrate per mg protein per min at 50°C.

Appropriate control reaction mixtures where the enzyme source or the substrate was omitted were used as blanks throughout the work.

RESULTS AND DISCUSSION

Partial purification of glutaminase activity using preheated extracts and Sephadex G-100 column chromatography

Figure 1 demonstrates the elution diagrams of L-glutamine deamidating enzyme during fractionation of the extract proteins using Sephadex G-100 (Methods). As it appears from the diagram, the activity was detected in protein fractions with one peak. In addition, the highest specific activity recorded corresponds to about 230 fold purification (Table 1).

Effect of pH

Data presented in Figure 2 shows that when partially purified enzyme was incubated with L-glutamine, as a substrate, at different pH values, the enzyme was somewhat stable in the pH range of 7.5-8.5 Tris- HCl. A rapid decline of the enzyme activity was observed on both sides of the pH optimum.

Testing the glutaminase activity in some buffer systems including, phosphate, Tris-HCl and borate revealed data in Table 2 indicating that Tris-HCl buffer was somewhat more suitable for the activity. Also testing the activity at different molarities of the Tris-HCl buffer (from 0.04 to 0.4) showed that optimum activities were obtained at a concentration of 0.16-0.2 mol/L.

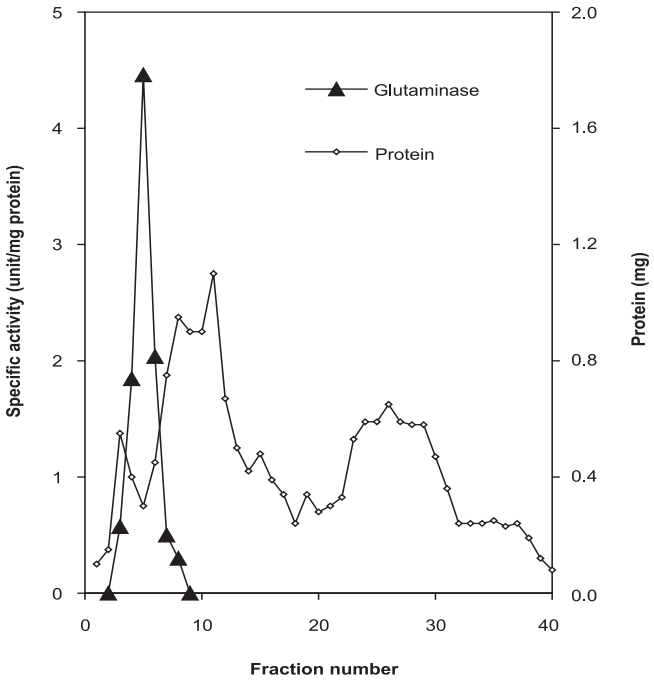

FIGURE 1. Elution diagram of glutaminase using Sephadex G-100 column chromatography.

TABLE 1. Partial purification of *P. politans* glutaminase.

Purification step	Total enzyme (U)	Total protein (mg)	Specific activity (U/mg protein)	Purification (fold)	Recovery (%)
Crude extracts	175	300	0.58	-	100
Heating	159	240	0.66	1.14	90
Sephadex G-100	44	0.33	133	230	25

Temperature-activities relationships and heat inactivation kinetics of the partially purified glutaminase

Figure 3 demonstrates the pattern of dependence of L-glutamine deamidation on the temperature of the reaction. From this figure, it appears that L-glutamine was highly deamidated at 60°C. In respect to this, when the partially purified enzyme was exposed in the absence of the substrate to 60°C for 30 min, the activity was stable. While it lost 30 and 55% of its activity when exposed to 70 and 75°C respectively at the same time, and a complete loss occurred when it was exposed to 80°C after 10 min, as depicted in Figure 4. It is also worthy of mention that both the extracts and the pooled fractions retained their activities when kept in the freezer for some months without thawing then freezing.

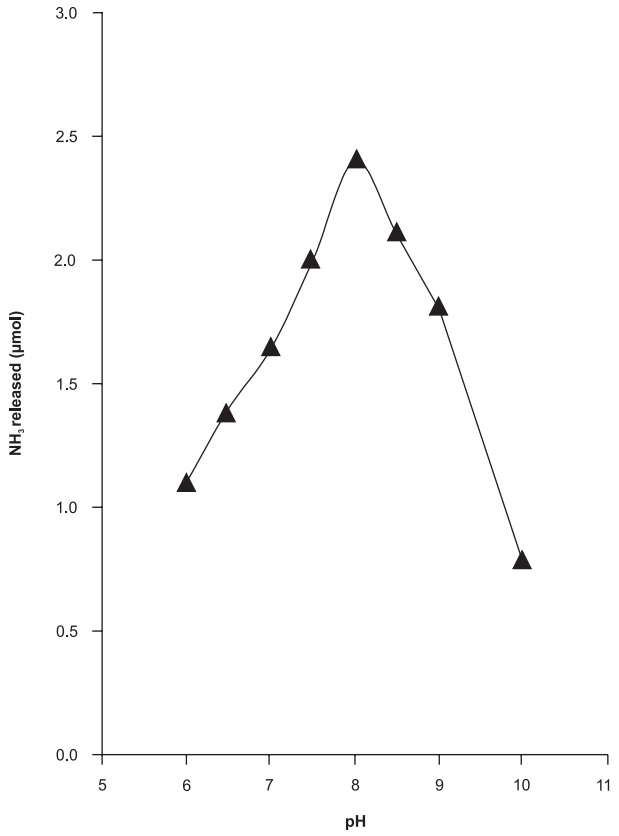

FIGURE 2. Dependence of the L-aglutaminase on the pH of the reaction. Reaction mixture contained (in 1 mL vol): substrate, 10.0 μmol; extract protein, 100 μg. ; buffer, 80 μmol (Tris-acetate pH 6-9, carbonate bicarbonate, pH9-10); time of the reaction, 30 min and temperature, 60.

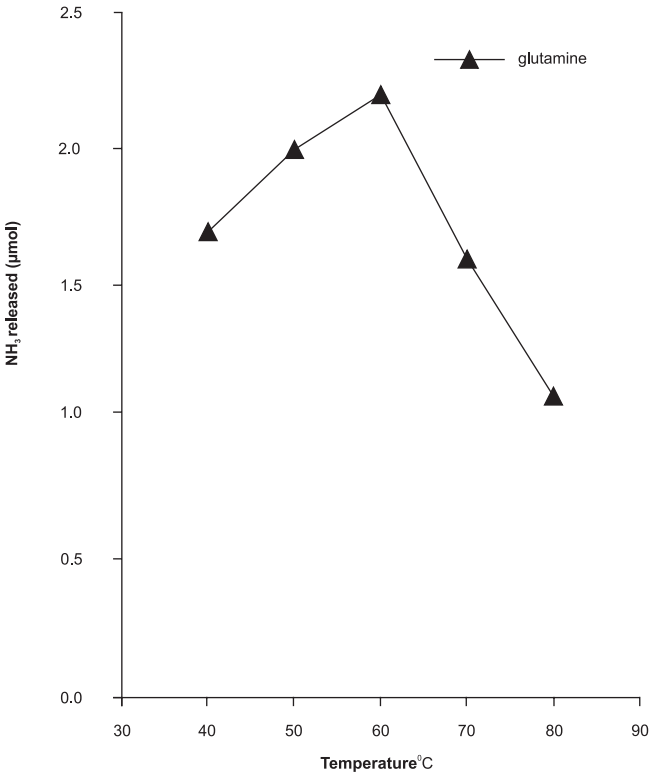

FIGURE 3. Dependence of the L-glutamine deamidating activity on the temperature of the reaction. Reaction mixture contained: Substrate, 10 mol; buffer, 80 mol Tris-HCl pH8; protein, 100 μg; temperature, as indicated; total vol.,1 mL; time, 30 min.

TABLE 2. Effect of nature buffer on glutaminase activity.

Buffer system	Activity (%)
Tris-HCl	100
Phosphate	73
Borate	68

Reaction mixture contained (in 1 mL vol): substrate, 10 μmoL; extract protein, 100 μg; buffer, at pH 8, 80 μmol; temperature, 50°C and time, 30 min.

Incompletion of L-glutamine deamidating reactions

When L-glutaminase was incubated with partially purified enzyme at the optimum conditions reached above, only about 26% of L-glutamine was converted into products (Figure 5), this may be due to product inhibition.

Inhibition of L-glutamine deamidating activity by the products of the reaction

Incompletion of the deamidation reaction observed in Figure 5 led to the suggestion that it could probably be due to product inhibition. Substantiation of this assumption can be observed from results of Figure 6 which show that addition of glutamic acid to the reaction mixture containing L-glutamine as substrate at the beginning of the reaction resulted in partial inhibition of the product.

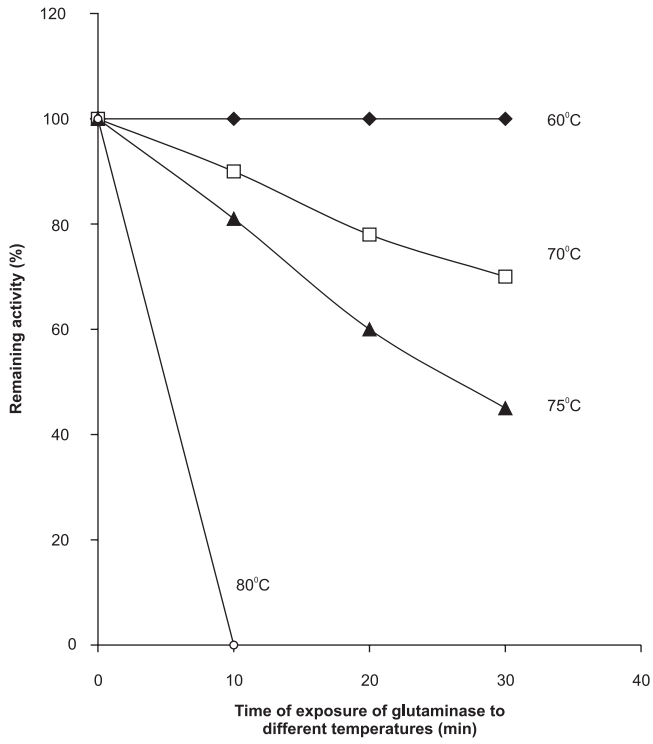

FIGURE 4. Heat inactivation kinetics of partially purified glutaminase. Reaction mixture contained: Substrate, 10 μmol; buffer, 80 mol Tris-HCl pH8; protein, 100 μg; temperature, 50°C; total vol.,1 mL; time, 30 min.

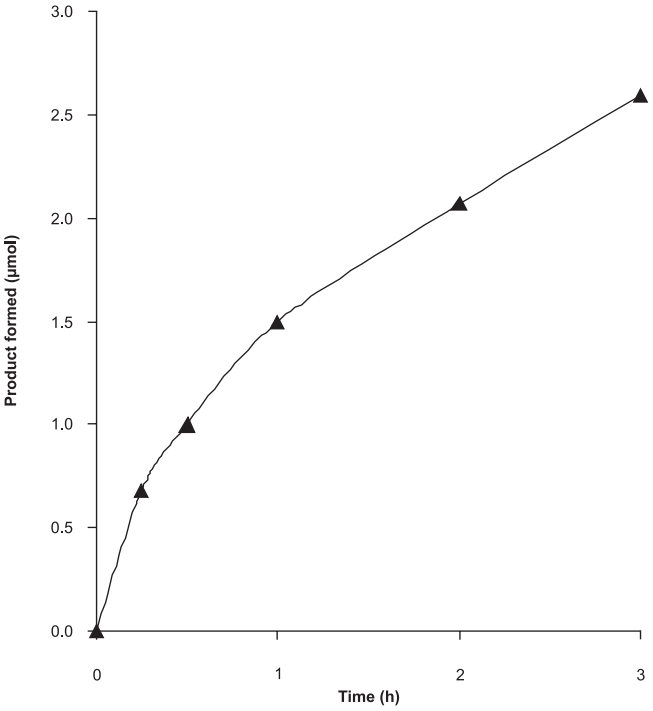

FIGURE 5. Incompletion of L-glutaminedeamidation. Reaction mixture contained: Substrate, 10 μmol; buffer, 80 mol Tris-HCl pH8; protein, 100 mg; temperature, 50°C; total vol.,1 mL; time,as indicated.

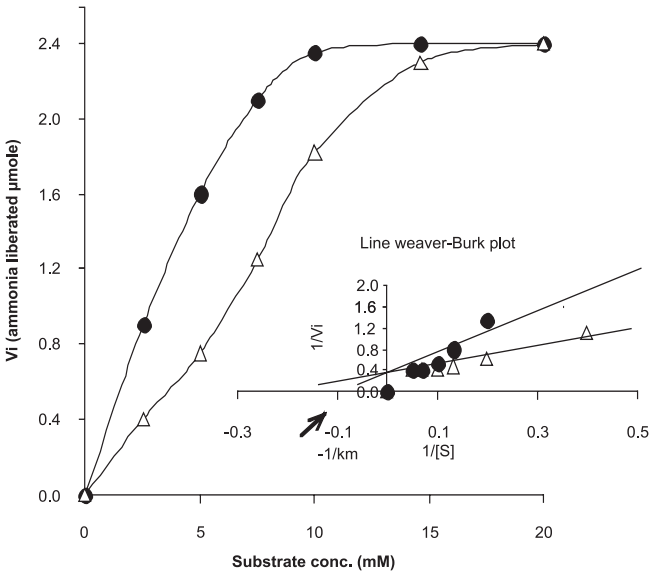

FIGURE 6. Substrate saturation kinetics of the glutaminase in the presence and absence of glutamic acid.

Determination of the type of inhibition exerted by glutamic acid

The plot of partially purified enzyme activity *versus* glutamine concentration in the absence and presence of glutamic acid clearly shows the significant inhibition in glutaminase activity. From Figure 6 it was found that the enzyme was inhibited in a competitive manner by glutamic acid. The apparent values of K_m and K_i were calculated from the Line weaver Burk plot and found to be 7.5 and 39 mol/L, respectively.

Substrate specificity of the partially purified glutminase deamidase(s)

Data of Table 3 shows that the amidohydrolase(s) of the pooled fractions exhibited activities only with L-glutamine and L-asparagine and their corresponding D isomers out of the tested compounds such as nicotinamide, acetamide and nicotinamide adenine dinucleotide. The rate of hydrolysis of D-glutamine and D-asparagine was about 30 and 37% of the rate for corresponding L-isomers, respectively.

Types of glutaminase reactions

The family of amidohydrolase that catalyzes the deamination of glutamine contains two classes. The first class includes glutaminase, which is highly specific for glutamine and catalyzes the hydrolysis of glutamine to glutamic acid. The second class contains the enzyme that is less specific and catalyzes the hydrolysis of glutamine to glutamic acid and asparagine to aspartic acid with similar efficiency [Nandakumar *et al.,* 2003]. Data cited in Table 3 suggests that *P. Politans* deamidating activity is considered as the second type.

Effect of some compounds on glutaminase activity

Results of Table 4 show that the L-glutaminase activity was enhanced in the presence of Mn^{++} and Mg^{++} and severe-

TABLE 3. Substrate specificity.

Compounds	Formed (μmol)	Hydrolysis (%)
L-asparagine	1.06	100
D-asparagine	0.4	37
L-glutamine	0.89	83
D-glutamine	0.32	30
Nicotinamide	0.0	0.0
Nicotinamide adenine dinucleotide	0.0	0.0
Acetamide	0.0	0.0

Reaction mixture contained (in 1 mL vol): substrate, 10 μmol; extract protein, 100 μg; buffer, Tris- HCl, pH 8, 80 μmol; temperature, 50°C and time, 30 min.

TABLE 4. Effect of some compounds addition on *P. politans* glutaminase.

Compound (10^{-2}M)	Relative activity (%)
None	100
$MnCl_2$	135.6
$Ca\ Cl_2$	65.5
$HgCl_2$	70
$MgSO_4$	127.8
$CuSO_4$	54
Na F	41.1
NH_3	65.6
$BaCl_2$	62
2-Merceptoethanol	92
Iodoacetate	94

Reaction mixture contained (in 1 mL vol.): substrate, 10.0 μmol; protein, 100 μgm.; Tris-HCl buffer at pH 8, 80 μmol; temp, 50°C; incubation period, 30 min and conc. of the added compounds, 20 μmol.

ly inhibited in the presence of Hg^{++}, Ca^{++}, Cu^{++}, Ba^{++} and F^{+}, while the enzyme was not inhibited by addition of 2-merceptoethanol and iodoacetate. Since iodoacetate is known to inhibit enzymes that have the SH group in their active sites, this suggests that no sulphyhdryl group participates in the enzyme activity. Addition of any of the products of the reactions, as glutamic acid and NH_3, at a concentration two fold that of the substrate, was found to have an inhibitory effect on any of the activity, which is in accordance with what has been suggested from data presented in Figure 5 and in the study reported [Ali & Elsayed, 2006].

Salt tolerance of glutaminase

As shown in Figure 7, a gradual reduction in glutaminase was observed by increasing the NaCl concentration. Glutaminase activity decreased 5%, 10% and 20% at 5%, 10% and 15% NaCl respectively. The residual enzyme activity was established at about 75% from its original activity at a range from 15-30% NaCl.

From these results, it can be suggested that the extracts of *P. politans* contain constitutively expressed amidohydrolase capable of hydrolyzing each of glutamine and asparagine at pH 8. According to substrate specificity, the *Penicillium politans* extracts are assumed to contain one amidohydrolase having dual specificity towards both L-glutamine and L-asparagine. This assumption was based on a number of given criteria which suggest that one enzyme was involved in the hydrolysis of the two amino acids. The *Penicillium politans* enzyme was similar to the glutaminase-asparaginase of the filamentous fungi found in each of *Aspergillus niger* $NRRl_3$ and *Penicillium chrysogenum*, which has been reported by [Elzainy & Ali, 2006]. These enzymes have constitutive nature and have been found in mats grown on a synthetic medium containing glucose plus mineral salts only.

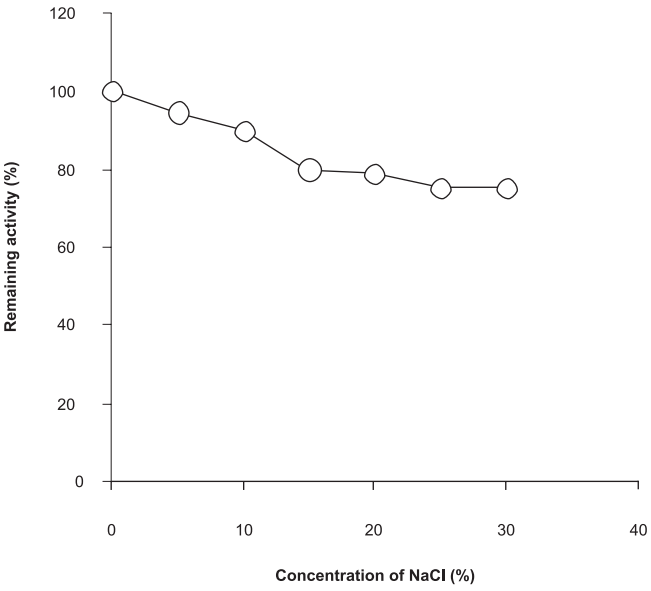

FIGURE 7. Effects of NaCl on glutaminase activity. Reaction mixture contained: Substrate, 10 μmol; buffer, 80 mol Tris-HCl pH8; protein, 100 μg, temperature, 50°C; total vol., 1 mL; time, 30 min, concentration of NaCl, as indicated.

On the other hand, amidases from strains of *Pseudomonas fluorescens* and *Pseudomonas putida* were induced when grown on acidic amino acids and their amides, even when supplied as the sole source of carbon and nitrogen [Sonawane *et al.,* 2003], the fungal two glutaminases from *Rhizobium etli* [Huerta-Zepeda *et al*., 1997], were detected in cells grown in the medium containing inducers for the enzyme biosynthesis such as glutamine and, also a *Rhizobium etli* Tn*5* insertion mutant, LM01, was selected for its inability to use glutamine as the sole carbon and nitrogen source [Calderon-Flores *et al.*, 2005].

Constitutively of the fungal enzyme indicates that it plays an essential role in maintaining the balance between glutamine and glutamate as well as between asparagine and aspartate, thus, regulating the intracellular concentrations of these four amino acids during the dynamics of cell metabolism [Huerta-Saquero *et al*., 2001].

Obtaining higher activity of the deamidating enzyme with L-asparagine than with L-glutamine, exposure of substrate specificity it suggested that *P. politans* deamidating activity is considered as the second type [Nandakumar *et al.,* 2003].

The maximal pH activity of the *P. politans* amidohydrolase with L-glutamine was almost similar to pH-activity profiles with the two amino acids on using extracts of either *Penicillium chrysogenum* or *Aspergillus niger*, however, optimum activities of extracts of the *Penicillium chrysogenum* was observed at pH 6-7 while the optimum pH of *Aspergillus niger* was at 8.

Glutaminase from *P. politans* is inhibited with addition of $CuSO_4$. However, Bhattacharya *et al.* [2001], reported that the presence of Cu with the enzyme, amplified the antineoplastic response by improving anti-angiogenic potential and hematological status of the tumor bearing host. Therefore, Cu-glutaminase combination strengthened the hypothesis that together they may provide a better therapeutic regimen in experimental mice tumor model.

Recording product inhibition for the *P. politans* glutaminase on addition of any of the two products of each reaction to the corresponding reaction mixture is similar to what has been reported for the enzyme from *Acinetobacter glutaminasificans* [Steckel *et al.,* 1983] that L-glutamate and L-aspartate showed competitive inhibition with respect to L-glutamine or L-asparagine, as a substrate, in contrast to the analogous enzyme of an *Achromobacter* soil organism [Roberts *et al*., 1972].

Glutaminase from *P. politans* showed stability in about 75% of its original activity, in the presence of 15-30% NaCl. These results indicate that the enzyme has a higher salt tolerance and thermal stability than *A. oryza* glutaminase [Yano *et al*., 1988] and it is similar to glutaminase from *Stenotrophomonas maltophilia* [Wakayama *et al.,* 2005], a model reaction of Japanese soy sauce fermentation, which exhibited high ability in the production of glutamic acid compared with glutaminases from *Aspergillus oryzae* [Thammarongtham *et al.,* 2001]. Glutaminases from marine isolates *M. luteus* K-3 and *B. subtilis* are shown to be highly salt-tolerant: tolerant to up to 16 and 25% of NaCl, respectively [Moriguchi *et al.,* 1994; Kennedy *et al.,* 2001; Madern *et al.,* 2000]. Glutaminase from *P. politans* is a stable at high temperatures as yeast enzyme from *Cryptococcus nodaensis* [Sato *et al.,* 1999] and

as the thermostable glutaminase activity in six thermophilic strains of *Pseudomonas* is reported by Rath & Subramanyam [1996], that their activities were observed even at a temperature of about 80°C.

CONCLUSION

The extracts of *P. politans* contain intracellular constitutive amidohydrolase having dual specificity of hydrolyzing each of L-glutamine and L-asparagine. The optimum deamidation of the enzyme was recorded at pH 8 and 60°C. The enzyme was inhibited by addition of glutamic acid or NH_3 to the reaction mixture and the glutamic acid inhibition was a competitive type. The enzyme was stable at high temperatures and had a high salt tolerance, which would significantly enhance its applications in the food industry. The present basic study may indicate that the properties of the *P. politans* enzyme could be in favor of its possible utilization, after further extensive studies, as an antitumor therapeutic agent.

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Received September 2008. Revision received December 2008 and accepted February 2009.