Activities and kinetic characteristics of glutamine synthetase from *Penicillium cyclopium*

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Abstract - The level of glutamine synthetase (GS; EC 6.3.1.2) obtained as a function of culture age of *Penicillium cyclopium* showed that GS activity increased continuously up to 120 hours after which the enzyme activity started to decline. Glutamine synthetase from *P. cyclopium* was purified to homogeneity by ammonium sulphate, diethylaminoethyl (DEAE)-cellulose and Sepharose 4B. The purified enzyme showed a single band. The pH optima for both the biosynthetic and transferase activities of the enzyme were 8.2 and 8.5, respectively. Various products such as 5'-AMP, tryptophan, alanine, glycine, and histidine inhibited both reactions of GS. However, ADP activated the transferase reaction and inhibited the biosynthetic one. The K_m values for L-glutamine were 1.4 and 5.6 mM in the biosynthetic and transferase assays, respectively. The divalent metal ion is necessary for the activity of the enzyme. Mn^{2+} was the most effective metal ion for transferase activity however, Mg^{2+} was the most effective metal ion for biosynthetic activity. Ca^{2+} and Mn^{2+} strongly inhibited Mg^{2+}-supported biosynthetic activity. GS was quite stable in tris (hydroxymethyl) aminomethane-HCl buffer (pH 7.5) containing ethylenediaminetetraacetate (EDTA), $MgCl_2$ and 2-mercaptoethanol (2-ME).

Key words: Glutamine synthetase, *Penicillium cyclopium*, purification, characterisation.

INTRODUCTION

Glutamine synthetase (GS; EC 6.3.1.2) plays a central role in ammonia assimilation in bacteria (Bespalova *et al*., 1999; Yadav *et al.,* 1999; Spinosa *et al*., 2000; Jukim *et al*., 2002; Kameya *et al*., 2006), fungi (Mash *et al*., 1997; Rudolfova and Mikes, 1997; Kersten *et al*., 2000) and plants (Guerrero and Lara, 1987; Ortega *et al*., 1999; Clemente and Marquez, 1999; Oliveira and Coruzzi, 1999; Carvalho *et al*., 2000) by collaboration with glutamate synthase (EC 1.4.7.1) (El-Shora, 2001).
GS is one of the key enzymes in nitrogen metabolism since it catalyses the synthesis of glutamine from glutamate according to the following reaction:

glutamate + NH_4^+ + ATP $\rightarrow$ glutamine + ADP + Pi + H^+
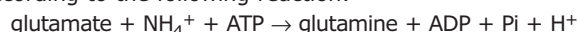
while glutamate synthase catalyzes the reductive transamidation from glutamine to α-ketoglutaric acid that assimilates one molecule of ammonium nitrogen (MeMaster *et al*., 1980; Brun *et al*., 1995; Carvalho *et al*., 2000; Mash *et al*., 2000; Liorca *et al*., 2006).

The incorporation of ammonium nitrogen into carbon skeletons takes place a key junction between nitrogen and the carbon metabolism. Although it does not directly link amino acids with the carbohydrate metabolism, it influences their connection by regulating the glutamine/α-ketoglutarate ratio. GS has been purified and well characterised from other sources (Merida *et al*., 1990; Brun *et al*., 1992; Shatters *et al*., 1993; Palaniappan and Gunasekeran, 1995; Mash *et al*., 2000; Kameya *et al*., 2006). All of these GSs were quite similar in molecular mass and the requirement of divalent metal ions for enzyme activity.

In view of the important role of GS in nitrogen metabolism of all organisms, an ever-interesting attention of investigators is being attracted for studying its reaction mechanism, regulation, and number of active sites. The information on the kinetic properties of GS from fungi particularly *Penicillium cyclopium* is very little. Therefore, the present investigation aimed to isolate, purify and characterise the GS from *P. cyclopium*.

MATERIALS AND METHODS

Growth of organism. *Penicillium cyclopium* was grown on a liquid medium containing the following components: 2% corn steep liquor, 1.2% $(NH_4)_2H_2PO_4$, 0.07% KCl, 0.05% $MgSO_4·7H_2O$, 0.001% $FeSO_4·7H_2O$; pH 4.5. The liquid

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culture was usually grown in 250 ml Erlenmeyer flasks with 100 ml medium at 27 °C in an orbital incubator for 3 days. Culture was inoculated from stocks kept on malt extract agar plates.

Enzyme extraction. Freeze dried mycelium to be assayed for GS activity was pulverized with an electric mixer in an extraction buffer (100 mM acetate buffer, pH 4.7, 5 mM dithiothreitol). Extract was filtered through gauze and clarified by centrifugation at 2795 x g for 20 min at 4 °C. The resulting supernatant was called the crude extract.

Purification of GS. The purification of GS was carried out according to El-Shora (1995). All experimental work during extraction and purification of the enzyme were carried out at 0-4 °C. The crude extract of GS was prepared and the enzyme was precipitated with solid ammonium sulphate in levels between 40-60% saturation. The precipitate was dissolved in 10 ml of extraction buffer and dialysed against this buffer before loading onto a DEAE-cellulose column (1 x 24 cm) equilibrated with the same buffer. The column was washed with the same buffer and the enzyme was eluted with the buffer containing 0.1 M NaCl. The fractions containing the bulk of GS activity were pooled and further purified on a Sepharose 4B column (1 x 24 cm) pre-equilibrated and eluted with 50 mM Tris-HCl (pH 7.8). The active fractions were pooled and stored at 4 °C.

Enzyme assays.

Transferase activity. The assay was carried out according to Shapiro and Stadtman (1970). The assay mixture consisted of 50 mM imidazole-HCl (pH 7.0), 0.2 mM ADP, 2 mM $MnCl_2$, 10 mM L-glutamine, 110 mM sodium arsenate, 30 mM NH_2OH and the enzyme solution in a final volume of 3 ml. The L-glutamine was omitted in the blank test. The reaction was started by adding NH_2OH (prepared freshly, and neutralised to pH 7.0 with NaOH) and incubated at 30 °C. The reaction was stopped after 15 min by adding 1.0 ml of a mixture of 10% $FeCl_3$ (in 0.2 N HCl), 24% trichloroacetic acid and 6 N HCl (1:1:1). The appearance of γ-glutamyl hydroxamate was measured by the increased absorbance at 540 nm.

Biosynthetic activity. The assay mixture for biosynthetic activity consisted of 30 mM imidazole-HCl (pH 7.0), 2.5 mM ATP, 50 mM L-glutamate, 20 mM NH_4Cl, 10 mM $MgCl_2$ and the enzyme solution in a final volume of 3 ml. The enzyme solution was omitted in the blank test. The reaction was started by adding the enzyme solution and incubating at 30 °C. It was stopped by adding 4 ml of $FeSO_4$ (0.8% in 0.015 N H_2SO_4, prepared freshly) after 30 min. The released inorganic phosphate (P_i) was determined by adding 0.5 ml of $(NH_4)_6Mo_7O_{24}$ (6.6% in 7.5 N H_2SO_4), and the absorbance was measured at 600 nm.

Protein determination. The protein content was estimated by the dye-binding method (Bradford, 1976). The bovine serum albumin (BSA) was used as the standard.

Electrophoresis and molecular weight determination. Sodium dodecyl-polyacrylamide slab gel electrophoresis was performed as described by Laemmli (1970). Protein standards employed were phosphorylase b (92500 Da), bovine serum albumin (66200 Da), ovalbumin (45000 Da), carbonic anhydrase (31000 Da), and soybean trypsin

inhibitor (21500 Da). Following electrophoresis, proteins were stained with Coomassie brilliant blue.

The effect of divalent cations. To detect the effect of divalent metal ions on the enzyme activity, the purified enzyme was dialysed against 10 mM Tris-HCl buffer (pH 7.5) containing 2 mM EDTA and 10 mM 2-ME with three changes of the buffer. The requirement of divalent metal ions for the enzyme activity and the effects of various divalent metal ions on the Mg^{2+}-supported biosynthetic activity and Mn^{2+}-supported transferase activity were examined. $MgCl_2$, $MnCl_2$, $CoCl_2$, $CaCl_2$, $CuSO_4$, $FeSO_4$, $ZnSO_4$ and $NiSO_4$ were used as the source of divalent metal ions.

Stability of GS. The stability of GS activity was detected by incubating the enzyme at 40, 50 or 60°C for different time intervals. The enzyme was then cooled in an ice bath, and the residual activity was determined by transferase and biosynthetic assays. To detect the thermostability of the enzyme activity in the presence of stabilising ligands, the purified enzyme was dialysed against 10 mM Tris-HCl buffer (pH 7.5) with three changes of the buffer. The enzyme was then incubated at 50 °C in 10 mM Tris-HCl buffer (pH 7.5) with or without stabilising ligands. The transferase and biosynthetic activities of the enzyme were determined after 15 min of incubation.

RESULTS AND DISCUSSION

GS production of *Penicillium cyclopium* was increased continuously during the growth up to 5 days after which it started to decline (Fig. 1). The purification steps are summarised in Table 1. The enzyme was purified 127.1-fold with specific activity of 241 U mg^{-1} protein. The GS from *P. cyclopium* was purified to homogeneity by ammonium sulphate, DEAE-cellulose, and Sepharose 4B (Fig. 2). GS from different sources has been purified to homogeneity and characterised (Orr *et al.*, 1981; Florencio and Ramos, 1985; Blanco *et al.*, 1989; Merida *et al.*, 1990, El-Shora *et al.*, 1994b; El-Shora, 1995).
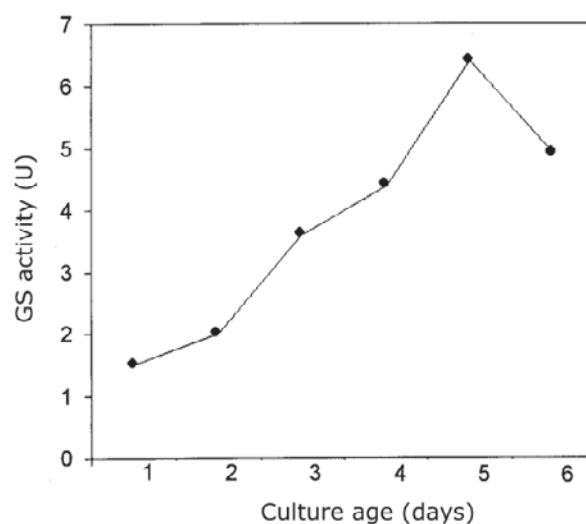

FIG. 1 - The relation between culture age and GS activity.

Table 1 - Purification of GS from *Penicillium cyclopium*.

Purification stage	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1} protein)	Purification factor	Yield (%)
Crude extract	320	588.0	1.9	1.0	100
(NH$_4$)$_2$SO$_4$ (40-60%)	75	410.0	5.5	2.8	69.7
DEAE-cellulose	11	328.1	29.8	15.7	55.8
Sepharose 4B	1.2	289.7	241.4	127.1	49.3

Table 2 - Effect of feedback inhibitors on GS from *Penicillium cyclopium*. Values are means ± S.E of three measurements.

Inhibitor (5 mM)	% Inhibition	
	Biosynthetic reaction	Transferase reaction
Control	0	0
L-Histidine	16.0 ± 0.9	28.1 ± 0.7
L-Tryptophan	38.0 ± 0.8	44.6 ± 0.4
Glycine	40.8 ± 0.5	53.3 ± 0.7
L-alanine	55.9 ± 0.7	71.0 ± 0.9
5'-AMP	63.3 ± 0.9	78.0 ± 0.2

The pH optima for both the biosynthetic (pH 8.2) and transferase (pH 8.4) activities of the GS (data not shown) were higher than that reported for the enzyme from different sources (Florencio and Ramos, 1985; Blanco *et al*., 1989; Merida *et al*., 1990; Shatters *et al*., 1993; El-Shora *et al*., 1994a; El-Shora, 1995; Palaniappan and Gunasekaran, 1995).

The end products metabolites such as 5'-AMP, glycine, L-alanine, L-tryptophan, and L-histidine inhibited both the biosynthetic and transferase reactions (Table 2). These results are in agreement with those of Bhatnagar *et al*. (1986) and Rudolfova and Mikes (1997). However, AMP inhibits both reactions and this is in harmony with the results of Zofall *et al*. (1996).
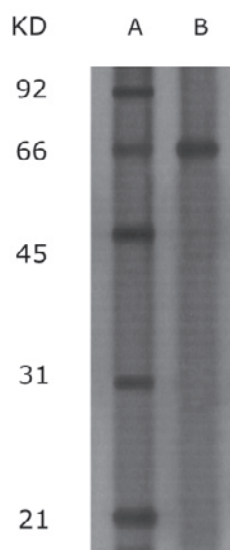

FIG. 2 - Electrophoresis for the purified GS from *Penicillium cyclopium* showing single band. (A) Markers and (B) pure GS from Sepharose 4B.

Thus, although GSs from different origins present structural similarities, their activities are regulated by different mechanisms (Orr and Haselkorn, 1981); feedback inhibition by various end products metabolites, divalent-cation induced conformational changes and covalent modification of the enzyme subunits (Bhatnagar *et al*., 1986; Yuang *et al*., 2001).

Kinetic properties of the purified GS were studied by analysing both the biosynthetic and transferase activities. The substrate affinities were calculated from kinetic measurements of the reaction rates by varying the concentration of one substrate with the other substrates in excess. The apparent K_m values (Fig. 3 and 4) were extrapolated from Lineweaver-Burk plots of the data obtained. In the biosynthetic reaction, the enzyme followed the Michaelis-Menten kinetics for L-glutamate with apparent K_m values of 1.4 and 5.6 mM in the biosynthetic and transferase reactions, respectively. These values are in consistent with those of Zofall *et al*. (1996). However, these values of K_m for L-glutamate were lower than that reported for other sources (Merida *et al*., 1990; Shatters *et al*., 1993; El-Shora *et al*., 1994a; Palaniappan and Gunasekaran, 1995). On the other hand, GS from *Rhizobium meliloti* showed higher K_m 13.3 mM for glutamate (Shatters *et al*., 1993).

The effect of divalent cations on both the biosynthetic and transferase reactions was studied at 10 mM in the reaction mixture of GS (Table 3). The reaction rate of transferase activity showed that Mn^{2+} was the most effective ion. These results are in harmony with those of Palaniappan and Gunasekaran (1995). Mg^{2+}, Co^{2+}, Ca^{2+} and Fe^{2+} were less effective, and the Ni^{2+} had no effect on transferase activity. The reaction rates of biosynthetic activity showed that Mg^{2+} was the most effective ion, and the next was Co^{2+}. At 10 mM of each ion, the observed order of effectiveness of different metal ions was $Mg^{2+} > Co^{2+} > Fe^{2+} > Mn^{2+} > Cu^{2+} > Zn^{2+}$. Ca^{2+} and Ni^{2+} had no effect on biosynthetic activity. However, Mg^{2+} showed the strongest stimulatory effect on the transferase and biosynthetic reactions (Zofall *et al*., 1996).
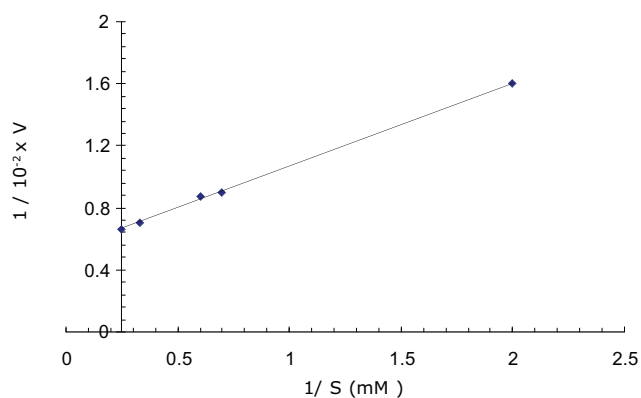
FIG. 3 - Effect of glutamate concentration on the biosynthetic reaction of GS. Double reciprocal plot of initial velocities with glutamate as the variable substrate.
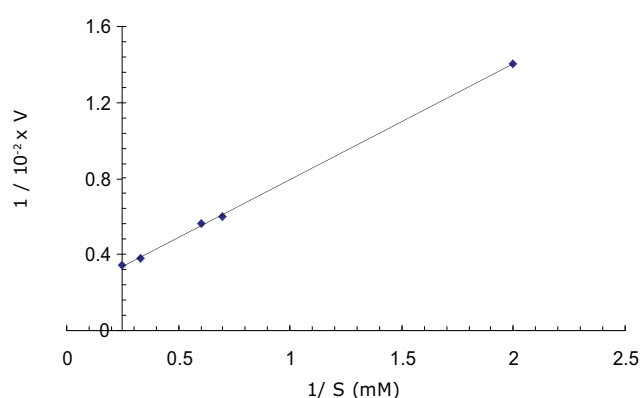

FIG. 4 - Effect of glutamate concentration on the transferase reaction of GS. Double reciprocal plot of initial velocities with glutamate as the variable substrate.

The effect of different divalent metal ions on the Mg-supported biosynthetic activity and the Mn^{2+}-supported transferase activity of the enzyme are given in Table 4. Ca^{2+}, Cu^{2+}, Fe^{3+}, Zn^{2+}, and Ni^{2+} showed strong inhibitory effects on biosynthetic activity. However, Co^{2+} showed a stimulatory effect on biosynthetic activity. These results are in consistent with those of Bespalova *et al.* (1999). Cu^{2+}, Fe^{2+} and Zn^{2+} strongly inhibited transferase activity but Co^{2+}, Ca^{2+}, and Ni^{2+} had less inhibitory effect on transferase activity.

It seems likely that the requirement of different concentrations of different divalent metal ions for enzyme activity might indicate that these metal ions stabilise different conformational states of the enzyme (Palmer, 1985).

Some reports have indicated that divalent metal ions play an important role in regulating the Mg^{2+}-supported biosynthetic activity of GS (Shapiro and Stadtman, 1970; Stacey *et al.*, 1979). The present study of divalent metal

Table 3 -   Effect of divalent cations on GS from *Penicillium cyclopium*. The values are means ± S.E. of three measurements. The contol samples did not contain any cation. The cations were used as chloride salt at 10 mM.

Divalent cation (10 mM)	GS activity	
	Biosynthetic reaction (% increase)	Transferase reaction (% increase)
Mg^{2+}	81.4 ± 0.8	38.0 ± 0.5
Co^{2+}	59.3 ± 0.4	21.4 ± 0.7
Fe^{3+}	39.0 ± 0.8	14.4 ± 0.8
Mn^{2+}	24.0 ± 0.4	70.0 ± 0.5
Cu^{2+}	19.5 ± 0.7	11.4 ± 0.4
Zn^{2+}	8.4 ± 0.9	3.8 ± 0.6
Ca^{2+}	5.1 ± 0.8	18.1 ± 0.7
Ni^{2+}	2.6 ± 0.4	3.0 ± 0.9

Table 4 -   Effect of different divalent cations on Mg^{2+}-stimulated biosynthesis and Mn^{2+}-stimulated transferase reactions of GS from *Penicillium cyclopium*. Values are means ± S. E. of three measurements. The control samples contain Mg^2 and Mn^2 for the biosynthetic and transferase reactions, respectively. The cations were used as chloride salt at 10 mM.

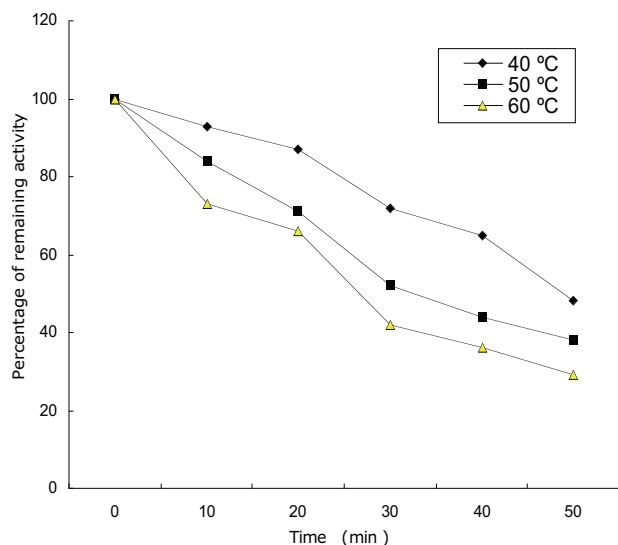| Divalent cation (10 mM)	GS activity	
	% activity in Mg^{2+}-stimulated biosynthesis	% activity in Mn^{2+}-stimulated transferase
Control	100	100
Co^{2+}	91.4 ± 0.6	46.4 ± 0.5
Fe^{3+}	29.0 ± 0.9	15.4 ± 0.7
Cu^{2+}	23.0 ± 0.1	11.6 ± 0.8
Zn^{2+}	38.0 ± 0.4	19.0 ± 0.9
Ca^{2+}	18.3 ± 0.8	55.0 ± 0.5
Ni^{2+}	48.6 ± 0.8	47.2 ± 0.4

FIG. 5 - Heat stability of GS at 40, 50 and 60 °C for the biosyn-
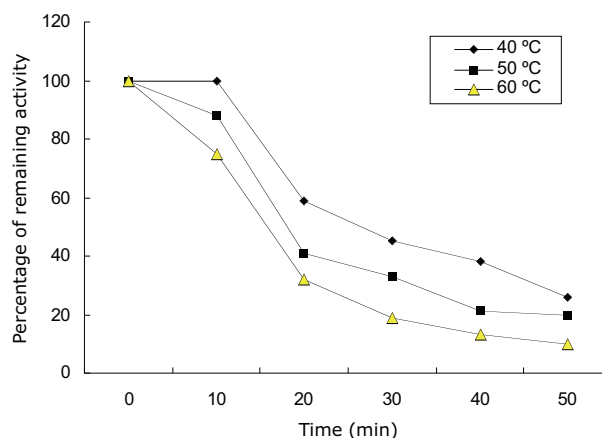thetic reaction.

FIG. 6 - Heat stability of GS at 40, 50 and 60 °C for the trans-
ferase reaction.

ions and the Mg^{2+}-supported biosynthetic activity of GS showed that Ca^{2+} and Cu^{2+} strongly inhibited such activity. In contrast, Co^{2+} showed a marked stimulating effect on GS activity of the biosynthetic reaction. This is in agreement with the results from *Anabaena* AC (Stacey *et al*., 1979), *Anabaena cylindrica* (Ip *et al*., 1983) and lupin nodule (Chen and Kennedy, 1985). It can be deduced that these metal ions compete for the Mg^{2+} binding site and have a higher binding constant, or they may have binding sites that are distinct or partially distinct from the Mg^{2+} binding site. Thus, the initial binding is followed by secondary reactions that vary with the kind of metal ion bound and yield a different conformational state for each metal ion to make different kinetic properties (Segal and Stadtman, 1972). This agrees with the suggestion of Ip *et al*. (1983) that GS may possess two types of metal ion binding sites: one catalytic site involved in substrate binding, and more than one regulatory site, to which activating and inhibitory metal ions bind.

The stability of the GS activity at various temperatures was determined for the biosynthetic reaction (Fig. 5) and the transferase one (Fig. 6). The enzyme was quite stable at 40 °C, but the enzyme activity decreased markedly at 50 and 60 °C. In contrast, the enzyme from *Nocardia asteroides* and *Rhizobium meliloti* (Shatters *et al*., 1993; Palaniappan and Gunasekaran, 1995) showed optimal temperature at 50 °C. The transferase activity of the enzyme appeared to decrease more rapid than the biosynthetic activity.

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