Indian J. Applied & Pure Bio. Vol. 41(1), 399-404 (2026). **ISSN: 0970-2091**

Extraction and Analysis of Phosphate Activated Glutaminase From Isolated Mitochondria of Sarcoma- 180 Tumour Cells

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Abstract

Phosphate-dependent glutaminase purified to homogeneity from isolated mitochondria of Sarcoma 180 tumour cells. The enzyme was purified 113 fold over the original cell free extract with a specific activity of 125μM/mg protein. The enzyme had an Mr of 64 KD as judged by chromatography on DEAE Sepharose ion exchange and Sephacryl S-300 column cgromatography. Two major immunoreactive peptides of Mr values of 64000 and 56000 were found by immunoblot analysis using anti-(rat kidney glutaminase) antibodies. The activity of the purified tumour glutaminase was maximal at pH 8.0. The concentration dependence for both phosphate and glutamine were sigmoid when assayed at pH 8.0. Glutaminase enzyme from S-180 tumour gives its maximum activity at 100mM potassium phosphate buffer which corresponds with the phosphate dependence of glutaminase purified from EAC tumour cells. Glutamine concentration optima of the purified glutaminase is 0.2mM. The Km value of S-180 cell glutaminase enzyme is found to be low *i.e*. 0.83 than rat liver glutaminase (17mM).

Key words : Glutaminase; S-180; Km; DEAE Sepharose.

Investigation on glutamine catabolism is of considerable interest because of its link with neoplastic transformation. Bacterial glutaminase was shown to be effective in lowering the tumor burden with increased life span of the host. However, bacterial glutaminase is also known for its suppressive effect on normal lymphocyte blastogenesis[1], peripheral blood cell counts and hematocrit[2]. These adverse effects on normal cell systems limit the optimal use of this enzyme as an antitumor agent and these findings lead us towards thepurification of glutaminase enzyme from mammalian source which may have better the rapeuticefficacy with less anti-genecity. Our present paper reports the isolation and purification of phospahate dependent glutaminase from a highly malignant Sarcoma-180 (S-180) cell maintained in mice, and some molecular and kinetic characteristics of the enzyme.

Objectives :

The anticipated advantage of the use of mammalian glutaminase as antiglutamine chemotherapyis dueto the fact that glutaminase finds application as an anti cancer drug, by specific inhibition of tumor cells glutamine uptake, thereby checking the tumor growth.

Problems associated with bacterial glutaminase is the low pH optima and high Km value. Glutaminase from mammalian cell may therefore be considered as a better choice for its pHoptima is near physiological pH having lower Km value.

Inbred strains of male Swiss albino mice (6-7 weeks, 20± (SD) 2 gm] obtained from our animal facility,were kept in cage, and maintained on standard mousefeed (Lipton, Bombay, India) and tap water ad libitum. EAC and S-180 cells were maintained in our laboratory by serial i.p. transplantation of 6-7 week old Swiss mice (1 x 10^5 cells/mouse) for ascites tumor and by s.c. transplantatiom (2 x 10^5 cells/mouse) for solid tumor. Methylcholanthrene induced solid tumor was, also formed by administrating 2 mg of methyl-cholanthrene/ mouse subcutaneouslyin the groin region. The cells were taken out from the peritoneal cavity after 8 days of tumor inoculation for enzyme purification from S-180 tumor. All purification steps were performed at 0°C 4°C. S-180 tumor cellswere separated from the peritoneal fluid by centrifuging at 2000 rpm. Mitochondria were isolated according to the method of Moreadith and Fiskum (3). 5 x 10s tumor cells were suspended in buffer, consisting of 5 mM HEPES, 210 mM mannitol l,70 mM sucrose, 1mM EGTA and 20mM sodiumborate, pH- 8.0. Cells were then sonicated for 3 min with 30 sec. pulse and 30 sec. interval, then centrifuged at 1,00,000 x g for 1 hr. Supernatant was 40% saturated by ammonium sulphate. Precipitated protein was isolated by centrifugation. The pellet was resuspended in 200mM potassium phosphate buffer pH-7.0. and dialyzed against the same. The sample was then applied to DEAE Sepharose column previously equilibrated with 10mM potassium phosphate buffer,pH 7.4. The sample was then eluted with a linear potassium phosphate gradient buffer (10mM-300mM) at a flow rate of 20 mi/hr. Fractions showing glutaminase activity were pooled and applied to an affinity column of L-gluta- mine insolubilized on 4% beaded agarose, Cyanogen bromide activated, previously equilibrated with 200mM potassium phosphate buffer pH-8.0 with 2 M NaCl. The fractions containing glutaminase activity were pooled anddialyzed against PBS and kept at -20 degree celcius for future experiments. The protein bands were visualised using SDS-PAGE followed by silver staining. The protein bands were visualized using SDS-PAGE [4] followed by silver staining. Immunoblot analysis was carried out by Towbin *et al*.,[5] using antibody of rat kidney glutaminase and immunoprecipitant bands were visualised.

Table-1. Purification of Phosphate dependent glutaminase enzyme from S-180cell

Steps	Volume (ml)	Unit (μM/ml/ min)	Total unit	Protein (mg/ ml)	Speci-fic activity	Yield (%)	Purifi-cation fold
S-180cellmitochondria after sonication	400	10.4	4160	9.6	1.1	100	1
S-180cellafterultra centrifuge	295	12.5	3687	2.1	5.9	88.6	5.4
S-180cellafterion exchange column	200	12.7	2540	0.2	6.35	61.05	57.7
S-180cellafteraffinity column	50	37.5	1875	0.3	125	45.07	113.6

Table-1 shows the procedure for the purification of phosphate dependent glutaminase from S-180 tumor strain. The purification of the final preparation of S-180 cell was apparently achieved 113 fold. Purified enzyme after affinity column chromatography showed one protein stained band with approximate Mr value of 64000 on SDS-PAGE.

Fig. 1. Protein profile of DEAE Sepharose column chromatography of S-180cell

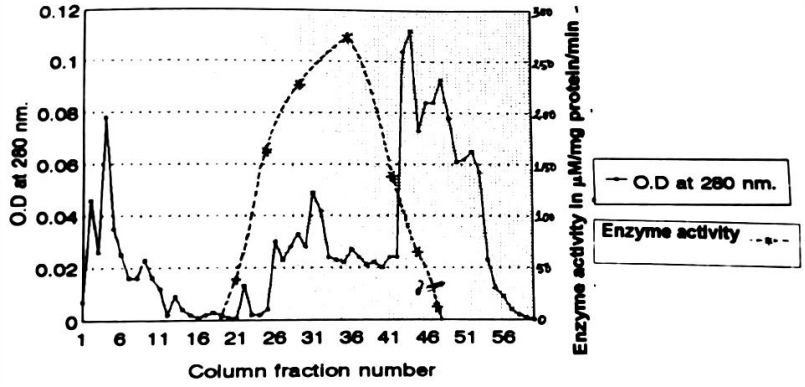

Figure 1 shows the protein profile of DEAE sepharose column chromatography of S-180 cell. Fraction 21 to 46 were pooled and applied to affinity column chromatography.

Fig. 2. Glutaminase enzyme activity of S-180cell and ascites fluid during S-180 carcinoma growth in mice

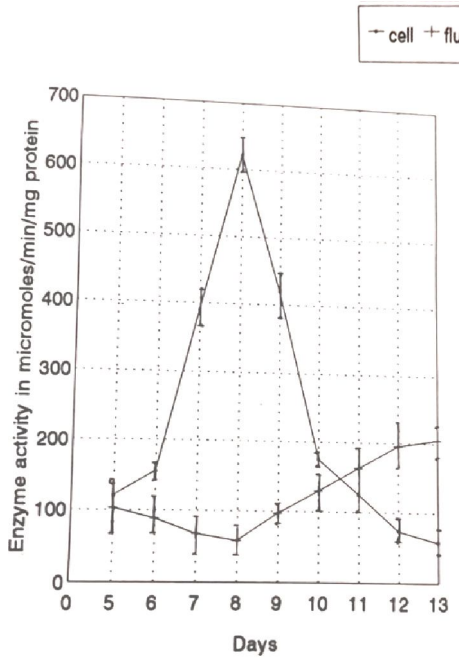

Figure 2. shows the glutaminase activity of S-180 cell and its ascites fluid in different days after inoculation of S-180 tumor in mice. The result demonstrates that maximum glutaminase activity from S-180 cell were obtained on the 8th day after tumor inoculation, so on the 8th day mice were sacrificed and tumor cells were taken for glutaminase purification.

Immunoblot analysis of glutaminase isolated from rat kidney and from S-180 tumor cells.

1. **S-180afterionexchangecolumn chromatography.**
2. **S-180afteraffinitycolumn chromatography.**
3. **Ratkidney mitochondria.**

Fig. 3

Fig. 3. In order to compare tumor glutaminase with the kidney type enzyme, immunoblot analysis of purified glutaminase from rat kidneys and S-180 cell (after ion exchange chromatography and affinity column chromatography) were carried out by using antibody against rat kidney glutaminase. The purified enzyme from rat kidney mitochondria isolated from S-180 cell exhibited one identical immunologically reactive band that seemed to correspond to the sub unit found in SDS-PAGE for the purified enzyme. This data support the idea that S-180 cell glutaminase is immunochemically identical with kidney type glutaminase.

Fig. 4. Glutamine concentration optima ofS-180(cell) glutaminase activity

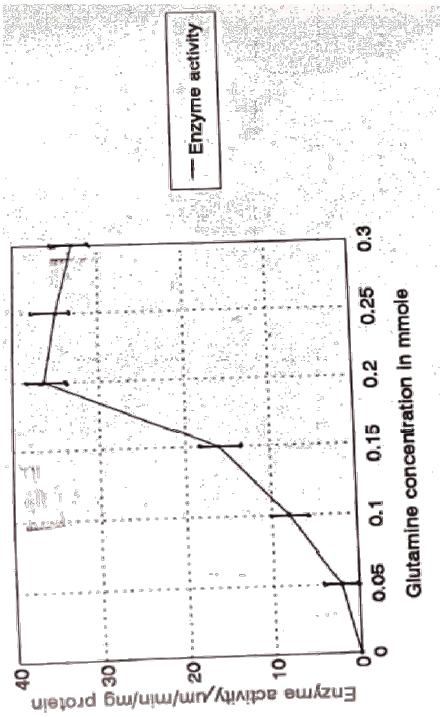

Figure 4. shows the glutamine concentration optima of S-180 cell glutaminase. It exhibits that glutaminase shows maximum activity in 0.2mM glutamine concentration.
Fig.5. pH optima of S-180 cell glutaminase

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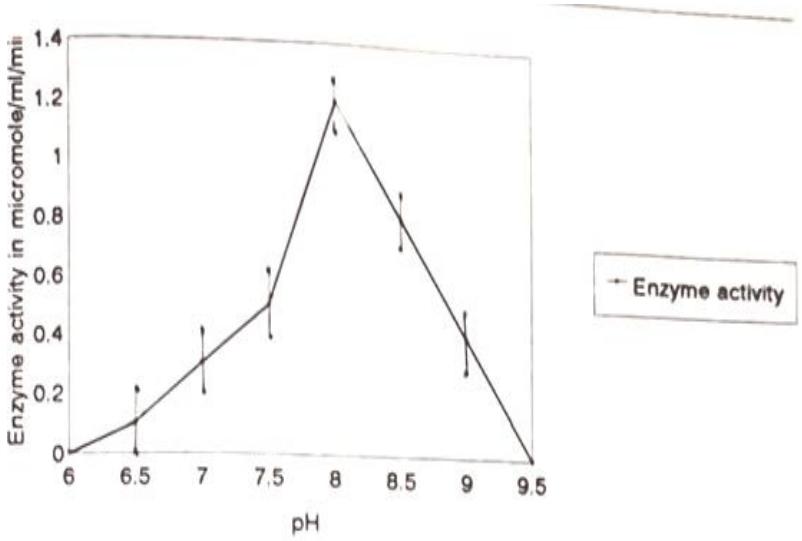

Figure 5 shows the pH optima of S-180 cell glutaminase. It shows the highest activity at pH 8.0.

Fig.6. Phosphate concentration optima of S-180(cell) glutaminase activity

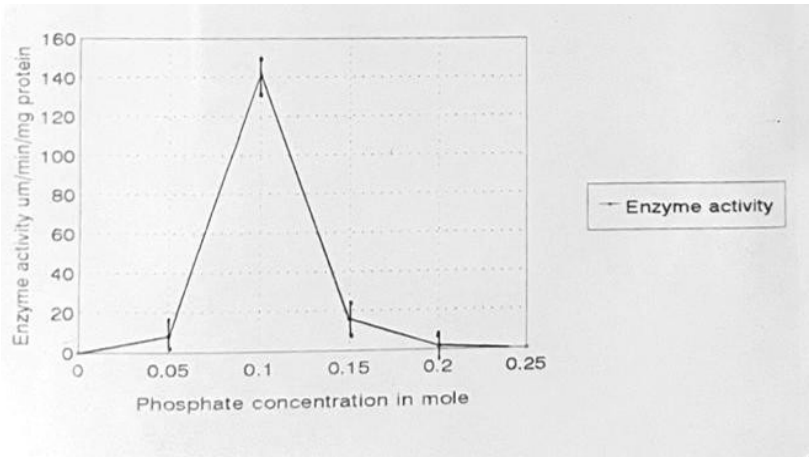

Figure 6. shows the phosphate concentration optima of S-180 cell glutaminase. It shows 0.1M phosphate for maximum activity.

Glutaminase enzyme was purified from highly malignant S-180 tumor strain. The enzyme was purified 113 fold over the original cell free extract with a specific activity of 125µM/mg protein. Quesada *et al*.,[5] purified phosphate dependent glutaminase from a highly malignant ascitic tumor strain. They purified the glutaminase enzyme upto 210 fold over the original cell freeextract. The purified enzyme from S-180 malignant cells gives a single band on SDS-PAGE. The result of immunoblot analysis suggests that the purified enzyme is of kidney type with a Mr value approximately 64KD. Glutaminase enzyme from pig brain has been reported to contain a single band of Mr 64KD by Shapiro *et al*.[6]. Quesada *et al*.[7] had shown that purified glutaminase from EAC tumor contains two bands of Mr value 64000 and 56000 on SDS-PAGE. They had used DEAE cellulose column and Sephacryl S-300 column for the purification of glutaminase. The activity of the purified tumor glutaminase was maximal at pH-8.0, the shape of the pH activity curve was very similar to thatof the enzyme isolated from rat liver. The concentration dependences for both phosphate and glutaminé were sigmoidal when assayed at pH 8.0. The S-180 tumor glutaminase exhibits lower Km (0.83) value than rat liver glutaminase (17mM). The purified enzyme from S-180 tumor with all these properties are used for future therapeutic study.

This work is supported by the grant from ICMR, New Delhi, India.

Conflict of interest :

Nil.

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