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ARTICLE

Localization of Phosphate Dependent Glutaminase in Ascites Fluid of Ovarian Cancer Patient

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Phosphate dependent glutaminase was purified from ascites fluid of ovarian cancer patients. The purified enzyme showed a final specific activity of 110 unit / mg protein with 72 fold purification and 21% yield. Purified enzyme gives one dark band of Mr ~65.5 KD and two light bands of Mr ~47.5 KD and ~45 KD respectively on 10% SDS-PAGE. One major immunoreactive band was found in trans-immunoblot analysis using antibodies against rat kidney and ascites fluid glutaminase raised in rabbit and mice respectively. Phosphate dependent glutaminase enzyme purified from mitochondria of malignant and non malignant ovarian tissue also showed bands of same molecular weight on 10% SDS-PAGE and gave same immunoreactive bands in trans-immunoblot like the purified glutaminase from ascites fluid. This result was confirmed by using the specific activity stain for glutaminase, which indicates that same enzyme activity is probably due to leakage of the same enzyme from malignant tissue into the ascites fluid. The purified enzyme from human peritoneal fluid showed a high specificity toward glutamine, therefore is a true glutaminase. Moreover, ascites fluid taken from patients of different age group with different stages of ovarian carcinoma revealed the presence of same glutaminase on 10% SDS-PAGE, and exhibited immunoreaction on ELISA, trans-immunoblot and dot immunoblot analysis. (Pathology Oncology Research Vol 6, No 3, 217–223, 2000)

Keywords: Ovarian cancer, ascites fluid, glutaminase.

Introduction

Glutamine is described as an important nutrient for rapid growing cells.[1-5,13] Its requirement is also very high in rapidly proliferating tumor tissue.[6-10,12] Glutamine provides multiple contribution to cellular growth by participating in protein, purine and pyrimidine metabolism. It is established that the level of glutamine metabolizing enzyme glutaminase increases with the fast progression of tumor and it helps in rapid utilization of glutamine by tumor cells.[11] Glutaminase from microbial source was well studied in cancer chemoprevention, specially in leukemia and lymphoma.[14] But, there is no report regarding the antineoplastic activities of mammalian glutaminase. Further there is report that phosphate dependent glutaminase enzyme is being leaked from the cell into the surrounding extracellular fluid and in turn, to the systemic circulation.[15] Therefore, an attempt has been taken to purify the glutaminase enzyme from ascites fluid of ovarian cancer patients and to characterize it biochemically and immunochemically.

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Materials and Methods

Ascites fluid and tissue samples were obtained from patients with different stages of histologically diagnosed ovarian carcinoma having age between 25–40, by the courtesy of Gynaecology department of our adjacent hospital wing.

Assay procedure

Phosphate dependent glutaminase was assayed by by endpoint determination of glutamate. Glutamate production was measured at 340 nm by monitoring NADH formation in the GLDH reaction, according to the method of Lund et al.[16] Protein was measured according to Lowry's method.[17]

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Table 1. **Purification of glutaminase enzyme from human ascites fluid of Ovarian cancer patient**

Step	*Vol (ml)*	*Unit (mM/ml/min)*	*Total unit (mM/ml/min)*	*Protein (mg/ml)*	*Spec. act.*	*Yield (%)*	*Fold Purification*
After filtration	60	8.5	510	5.6	1.51	100	1
After dialysis	10	24	240	1.4	17.14	47	11.35
After ion exchange column chromatography	200	0.9	180	0.03	30	35.47	19.87
After affinity column chromatography	100	1.1	110	0.01	110	21.57	72.85

Purification method

Ascites fluid of ovarian cancer patients were first centrifuged at 5000 r.p.m for 10 minutes. Then the supernatants were filtered through cheese cloth. Filtered samples were then subjected to 70% ammonium sulphate precipitation followed by dialysis in 200 mM (pH-7) potassium phosphate buffer. Dialysed samples were charged into DEAE Sepharose ion exchange column using a linear gradient of 10–300 mM potassium phosphate buffer (pH-7.4). Eluted samples were then applied to affinity column of L-glutamine insolubilized on 4% beaded agarose and activated by cyanogen bromide.

The ovary tissue (both malignant and benign) were minced first and then homogenized in media A containing 35 mM sucrose, 5 mM HEPES and 1 mM EDTA. The homogenized samples were then filtered through cheese cloth and centrifuged at 2500 r.p.m for 10 minutes. Supernatant was taken and centrifuged at 12,600 r.p.m for 5 minutes. Pellet was taken and resuspended in 10 mM potassium phosphate buffer (pH-8.0). Protease inhibitor was added and sonicated at 175 watt for 10 times with 15 seconds pulse and 30 seconds interval each time. Sonicated samples were then centrifuged at 28,000 r.p.m for 35 minutes and finally supernatants were suspended again in 100 mM potassium phosphate buffer (pH – 8.0) for purification through ion exchange and affinity column. Both the purification procedures were carried out at 4°C.

Mr estimation

Mr value of the sub units were estimated by calculating the Rf values, using known molecular weight markers, e.g. Carbonic anhydrase I (29 KD), glyceraldehyde dehydrogenase (36 KD), Ovalbumin (45 KD), Carbonic anhydrase II (47.5 KD), BSA (66 KD), Phospholypase B (97.4 KD), β-galactosidase (116 KD), Myosin (205 KD) on 10% SDS-PAGE. The method of SDS-PAGE is according to Laemmli (19). The 10% SDS-PAGE with ascites fluid from different patients was scanned under the gel scanner (Shimadzu Dual wave length flying spot scanner CS-9000).

Production of antibody in mice

The antisera against the purified glutaminase (ascites fluid of ovarian cancer patients) was raised in Swiss albino mice by subcutaneous injection of the enzyme emulsified with Freund's complete and incomplete adjuvant (SIGMA) alternately at 7 days interval. After 4th injection the blood was collected by cardiac puncture for collection of the antisera.[32]

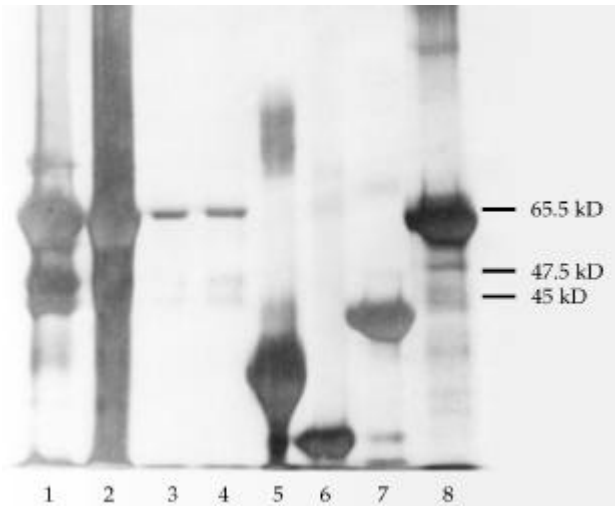

Figure 1. 10% SDS-PAGE of samples obtained after different steps of the purification procedure. The following samples were applied (1) after centrifugation at 5000 r.p.m and filtration (2) after ammonium sulphate precipitation followed by dialysis (3) & (4) after affinity column chromatography (5) ovalbumin (45 KD) (6) glyceraldehyde dehydrogenase (36 KD) (7) carbonic anhydrase II (47.5 KD) (8) bovine serum albumin (66 KD).

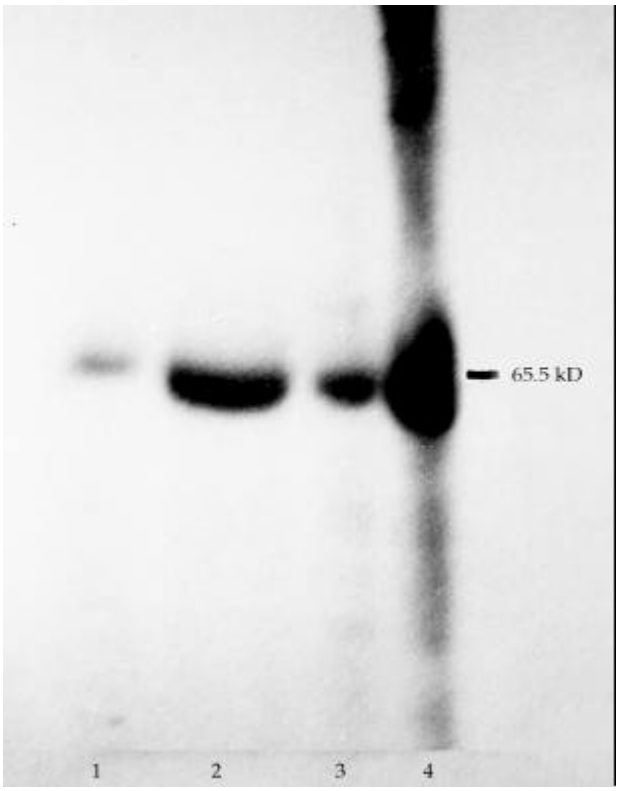

Figure 2a. Western blot analysis of glutaminase isolated from human ascites fluid and benign and malignant ovarian tissue. (1) Malignant ovarian tissue sample after affinity (2) Human ascites fluid sample after affinity (3) Benign ovarian tissue sample after affinity (4) Human ascites fluid sample after dialysis.

Immunoblot analysis

It was carried out essentially as described in Towbin's method.[20] Samples were subjected to 10% SDS-PAGE and then transferred to nitrocellulose paper. After blocking with BSA-Tween20-PBS solution the nitrocellulose papers were incubated with anti-rat kidney glutaminase antibody and also with the mouse primary antibody raised against purified glutaminase, for overnight at 4°C. Then they were incubated with their respective peroxidase conjugated antibodies for 1 hr. at room temperature. Staining was carried out by immersing the nitrocellulose papers into the solution containing 6 mg 4 Chloro-1 naphthanol in 20 ml methanol and 100 ml TRIS-HCl buffer (pH-7.6) with 0.1% (V/V) hydrogen peroxide.

Dot immunoblot analysis

This method has been performed according to Escribano et al.[33] The purified glutaminase was spotted on nitrocellulose paper and air dried. The paper was treated with the post fixative solution, washed with TBS and was incubated overnight at 4°C with mouse anti-glutaminase antibody. Then it was incubated with peroxidase labeled mouse second antibody for 2 hours at room temperature and color was developed after staining with the substrate solution containing 4 Chloro-1 naphthanol and hydrogen peroxide.

ELISA

This method was done according to Voller et al.[34] The purified glutaminase from different patients were added into the wells of a 96 well microlitre plate and incubated initially for 2 hrs. at 37°C followed by 12 hrs. at 4°C. After a thorough wash each wells were incubated with mouse antibody raised against the purified glutaminase for 2 hrs. at 4°C. After washing the wells were again incubated with peroxidase labeled mouse second antibody for 1 hr. at room temperature. Finally after washing the substrate solution containing ortho-phenyl-diamine and hydrogen peroxide was added to each well and O.D.were taken in ELISA reader at 490 nm after the development of colour.

Activity staining specific for glutaminase

7.5% native PAGE was prepared using 0.107 M Bistris (pH-7.12) as gel casting buffer. Two running buffers were used here. The upper electrode buffer (cathode) contains

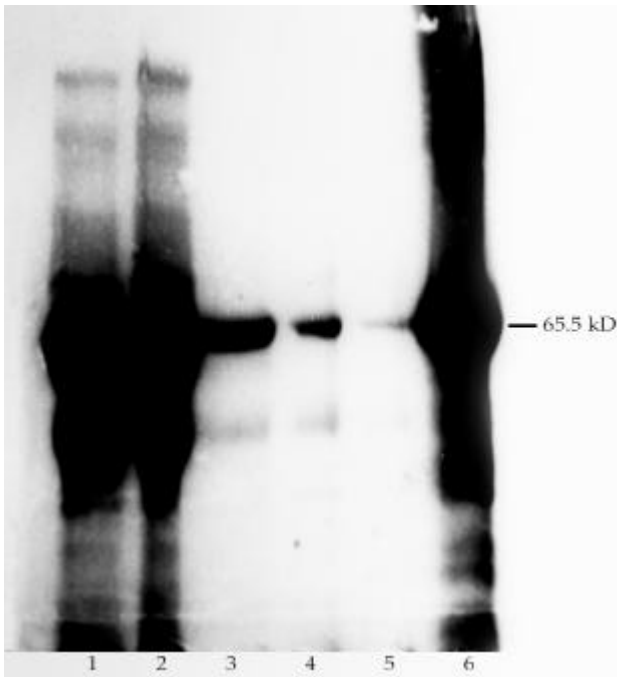

Figure 2b. 10% SDS-PAGE of samples obtained after different steps of the purification procedure. The following samples were applied (1) human ascites fluid after centrifugation at 5000 r.p.m and filtration (2) human ascites fluid after ammonium sulphate precipitation followed by dialysis (3) human ascites fluid after affinity column chromatography (4) benign ovarian tissue after affinity (5) malignant ovarian tissue after affinity (6) bovine serum albumin (66 KD).

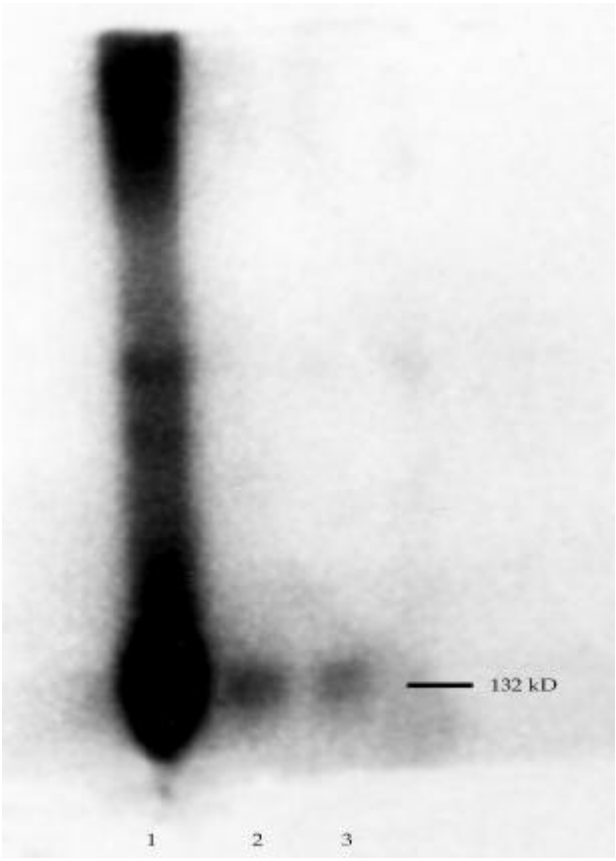

Figure 3. Activity staining specific for glutaminase using following samples on 7.5% native PAGE (1) human ascites fluid after dialysis (2) human ascites fluid after affinity (3) malignant ovarian tissue after affinity.

0.0198 M Bistris (pH-7.4) and lower electrode buffer (anode) contains 0.625 M Bistris (pH-6.28).

Gel was run at a constant current of 12-15 mA at 4°C for almost 3 hours. The gel was stained with freshly prepared glutaminase specific staining solution which contains L-Glutamine (15 mM), Bovine liver glutamate dehydrogenase (0.5 mg/ml), Potassium Phosphate buffer (100 mM, pH-7.1), NAD (2mg/ml), Phenazine methosulphate (0.04 mg/ml) and nitroblue tetrazolium (2 mg/ml).

Gel was immersed in staining solution and incubated at 37°C on a shaker for 5–20 min. The color reaction was stopped by rinsing the gel with distilled water and then stored at 10% acetic acid. The procedure followed here is according to Redbard's method.[30]

Determination of enzyme stability

The purified glutaminase after ion exchange chromatograph were incubated with different protease inhibitors, such as, 1mM PMSF (Phenyl Methyl Sulphonyl Fluoride), 20 μM Chymostatin and 10 μM Leupeptin. The stability of the enzyme was determined under these condition for 600 hrs. And *Figure 4* suggests that stability is greater in enzyme incubated with inhibitors than the enzyme alone. And from the result it is also evident that chymostatin acts as a strongest inhibitor followed by leupeptin and PMSF. The procedure is according to Heini et al.[29]

Statistical analysis

All results are confirmed by three different determination from three patients.

Results

Purification table (*Table 1*) shows the procedure of the purification of glutaminase enzyme from ascites fluid of ovarian cancer patient. The specific activity of the final preparation is increased to 110 unit/mg protein over the ultracentrifuged sample with 72 fold purification and 21% yield. One peak has been observed in the ion exchange chromatography in between fraction 19–29. The peak showed significant enzyme activity and eluted at ~100-150 mM eluting buffer. In 10% SDS-PAGE the purified enzyme protein after affinity column (*Figure 1*) gives one dark band of Mr ~65.5 KD and two light bands of Mr ~47.5 KD and ~45 KD, respectively.

Western blot analysis has been carried out with affinity samples of human ascites fluid ,malignant and benign part of neoplastic ovary, using anti-rat kidney glutaminase antibodies [kindly provided by Dr. N. P. Curthoys and method obtained as described by Haser et. al.[22]]. Here, all the affinity fractions (*Figure 2a*) exhibits bands of same molecular weight which seemed to corresponds with the bands found on 10% SDS-PAGE (*Figure 2b*). These data confirm that human ascites fluid as well as benign and malignant ovary tissue glutaminases are immunogenic and kidney type glutaminases, as they are

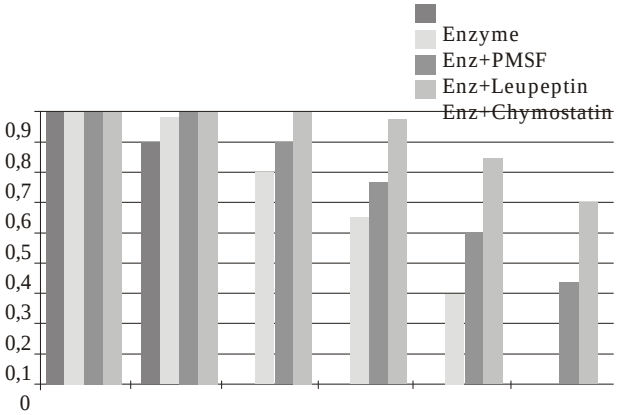

Figure 4. Enzyme stability of ascites fluid glutaminase from ovarian cancer patient.

immunoprecipitated by antibodies produced against isolated renal enzyme[21] as well as antibodies against ascites fluid glutaminase.

Specific activity staining for glutaminase gave same band in the affinity fractions of both human ascites fluid and malignant ovarian tissue on 7.5% native PAGE (*Figure 3*). Studies with ascites fluid glutaminase purified from different patients with ovarian carcinoma revealed the presence of same glutaminase in all of them. Trans-immunoblot analysis with purified glutaminase from 8 different patients gave bands at the same level (*Figure 5*) which corresponds with the bands on 10% SDS-PAGE (*Figure 6*). Dot immunoblot analysis with the same patients also revealed that mouse primary antibody raised against purified ascites fluid glutaminase reacted evenly with each patient's sample (not shown). ELISA with ascites fluid glutaminases from different patients showed higher value of optical density in ovarian carcinoma bearing individuals than the normal individual (*Figure 7*). Scanning of 10% SDS-PAGE at 410nm (by measuring the area of purified glutaminase in gel scan) revealed that purified glutaminases from different patients with various stages of ovarian carcinoma has a direct correlation between advancement of the disease and the level of glutaminase in the ascites fluid (*Table 3*).

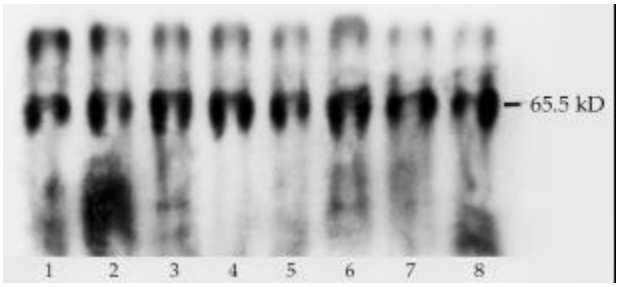

Figure 5. Western blot analysis of purified glutaminase from human ascites fluid. (1-8) purified glutaminases from patients with different stages of ovarian carcinoma.

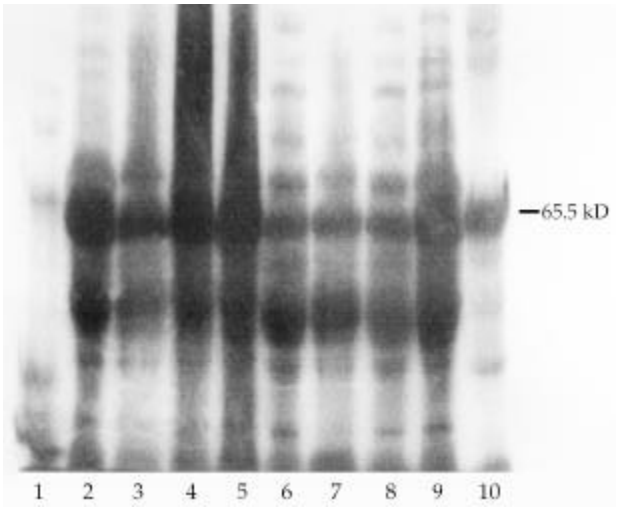

Figure 6. 10% SDS-PAGE of samples after dialysis. (1) A molecular weight marker mixture containing Carbonic anhydrase I (29KD), glyceraldehyde dehydrogenase (36KD), Ovalbumin(45KD), BSA(66KD), Phosphorylase B(97.4KD), (-galactosidase(116KD) and Myosin (205KD). (2-9) Purified glutaminases after dialysis from different patients. (10) BSA(66KD).

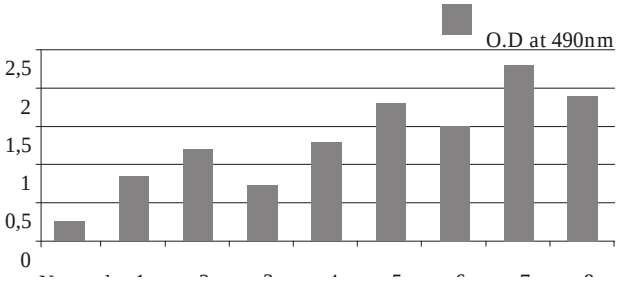

Figure 7. ELISA of purified glutaminase from ascites fluid of ovarian carcinoma bearing patients.

The purified glutaminase showed its maximum activity at pH-8.5. The shape of activity curve is very similar to that of the rat kidney glutaminase (pH-8.0). The enzyme is very specific for glutamine as it does not belongs to the group of enzymes which readily catalyses the hydrolysis of both asparagine and glutamine. Therefore, seems to be a typical glutaminase. This enzyme gives its maximum activity at 0.1 mM concentration of glutamine with a km value 0.5-1.0 .

Table 2. **Anion concentration optima of purified glutaminase from ascites fluid of ovarian cancer patient**

Anions	*Enzyme activity (in U/ml) at different concentrations (in mole)*					
	0.05	0.1	0.15	0.2	0.25	0.3
Phosphate	4.25	7.0	11.5	8.0	5.5	2.2
Borate	0.2	0	0	0	0	0
Succinate	0	0	0	0	0.1	0
Citrate	0	0.02	0	0	0	0

According to the *Table 2* the activation of the purified glutaminase is very much dependent on added phosphate and gives its maximum activity at 150 mM phosphate concentration. Other anions, such as, borate, citrate, succinate etc. at the same concentration unlike the phosphate failed to activate the enzyme.

Discussion

To our knowledge, we are the first to report localization, isolation and purification of kidney type phosphate dependent glutaminase enzyme from ascites fluid of ovarian can-

Table 3. **Relationship between glutaminase activity and different stages of ovarian carcinoma**

Number of patients	*Age of patients carcinoma*	*Stages of ovarian assay (μM/ml/min)*	*Purified glutaminase unit determined by enzyme assay (mM/ml/min)*	*Area specific for glutaminase*
1	35	II	123	21171
2	27	III	150	27220
3	38	II	111	16007
4	35	III	157	27334
5	30	III	173	32187
6	40	III	164	29539
7	36	IV	220	47857
8	25	IV	198	43208

cer patient. Previously kidney type phosphate dependent glutaminase has been purified from pig[25] and rat kidney[23] and also from pig,[26] rat[22] and cow brain.[24] Presence of kidney type glutaminase has also been reported in EAC cell and S-180 cell.[35] Kidney type glutaminase purified from EAC cell showed two bands of Mr value 64,000 and 56,000 respectively on SDS-PAGE[18] with a Km value 7.6. This finding is in agreement with the values previously reported by Olsen et. al[27] for pig kidney glutaminase. In contrast kidney type glutaminase from pig brain has been reported to contain a single polypeptide of Mr 64,000.[26] Haser et al[22] reported that kidney type glutaminase from rat brain contains two different peptides of Mr 65,000 and 68,000. Some weaker bands below Mr 61,000 was also observed, probably produced due to degradation of major peptides by the action of proteinases.[28] Rat kidney glutaminase show Km value ranging from 2–5. Here, in this kidney type glutaminase we found one major band of Mr 65,500 and two minor bands of Mr 47,500 and 45,000 respectively. So, this enzyme preparation might show higher glutamine clearance over the enzymes of other sources as it possess very low km value (0.5–1). The purified glutaminase was found to be stable for almost 25 days with protease inhibitors, thus provides more suitability to glutamine exploited chemotherapy. The same glutaminase purified from different patients exhibited that though it is present in all patients but its amount varies with the stages of ovarian carcinoma bearing individuals, which might be due to differences in release of glutaminase at different stages of cancer. It is already known that glutaminase productuion is proportional with the progression of tumor.[35,36] Therefore, at initial stages where tumor burden is small ovarian carcinoma showed lesser amount of glutaminase production than in the advanced stages. Whether this ascites fluid glutaminase was same as mitochondrial glutaminase of benign and malignant ovarian tissue glutaminase, specific glutaminase stain was performed on native gel. The result presented here showed that they are of same molecular weight. The mitochondrial glutaminase is released from tissue into ascites fluid is probably due to tumor necrosis or change in membrane permeability of cancer cells.[31] Thus this report concerns with mammalian glutaminase which can be purified easily and may be useful in cancer chemoprevention either alone or in combination.

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