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Antitumor activity of L-asparaginase from *Thermus thermophilus*

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L-Asparaginase (EC 3.5.1.1) was purified to homogeneity from *Thermus thermophilus*. The apparent molecular mass of L-asparaginase was found to be 33 kDa by SDS–PAGE, whereas by Sephacryl S-300 superfine column it was found to be 200 kDa, indicating that the enzyme in the native stage acts as hexamer. It is a thermostable enzyme and keeps all of its activity at 80°C for 10 min. The antiproliferative activity of the purified L-asparaginase from *T. thermiphilus* was tested against the following human cell lines: K-562 (chronic myelogenous leukemia), Raji (Burkitt's lymphoma), SK-N-MC (primitive neuroectodermal tumor), HeLa (cervical cancer), BT20 and MCF7 (breast cancers), HT-29 (human colon cancer), and OAW-42 (ovarian cancer). The antiproliferative activity of *T. thermophilus* enzyme was compared with Erwinase, the commercially available L-asparaginase from *Erwinia corotovora*. The potency difference between the two L-asparaginases was greater in HeLa and SK-N-MC than in other cell lines. The fact that L-asparaginase from *T. thermophilus* does not hydrolyse L-glutamine makes it advantageous for future clinical trials. [© 2001 Lippincott Williams & Wilkins.]

Key words: L-Asparaginase, antitumor activity, Erwinase, human cancer cell lines, *Thermus thermophilus*.

Introduction

L-Asparaginase (L-asparagine-amidohydrolase, EC 3.5.1.1) is the enzyme which converts L-asparagine to L-aspartic acid and ammonia. Interest in this enzyme arose a few decades ago when it was discovered that the antilymphoma activity of whole guinea pig serum was a result of the enzyme L-asparaginase.[1] Unlike normal cells, the malignant cells can only synthesize L-asparagine slowly and are dependent on an exogenous supply.[2] This sensitivity of the cells is related to the deprivation or the presence of small amounts of L-asparagine synthetase in contrast to non-sensitive neoplastic cells and physiological tissues.[3,4] Therefore, the clinical action of L-asparaginase is attributed to reduction of L-asparagine, since tumor cells are selectively killed by L-asparagine deprivation.[5] L-Asparaginases from various sources like *Escherichia coli*,[6] *Erwinia carotovora*,[7] *Serratia marcescens*[8] and *Vibrio succinigenes*[9] have been found to be pharmacologically active.

We have recently shown that L-asparaginase of *Tetrahymena pyriformis* is a multi-subunit enzyme exhibiting protein kinase activity as well.[10] Both native and dephosphorylated L-asparaginase show antiproliferative activity on three breast cancer cell lines (T47D, MCF7 and BT20) and on Walker 256 cells.[11]

L-Asparaginase has been used in combination with other anticancer drugs like azaserin,[12] vincristine[13] and methotrexate,[14] as well as with antagonists of glutamine or glutamic acid.[15]

L-Asparaginase therapy is complicated by immunological reactions and various side effects (acute pancreatitis, hyperglykemia, acute parotitis, etc.). L-Asparaginase-induced immunosuppression has been attributed to the glutaminase action possessed by the *E. coli* and *Erwinia* L-asparaginases.[16,17] Therefore, for the clinical studies (i) a glutaminase-free L-asparaginase and (ii) an L-asparaginase with less side effects will be more advantageous. Another limitation of L-asparaginase is the short plasma half-life, ranging from 2.5 h in mice to about 18 h in man.[18]

Enzymes that work either at extreme pH values or at high temperatures are now of biotechnological interest.[19,20] Therefore, purification of enzymes with these properties presents genuine commercial opportunities and a powerful tool for new therapeutic trials.

The present study provides evidence that L-asparaginase of the thermophilic bacterium *Thermus thermophilus*, which is a thermostable and glutaminase-free enzyme, presents antitumor activity against human cancer cell lines.

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Materials and methods

Materials

Tryptone and yeast extract were purchased from Difco (Detroit, MI). Phenyl sepharose CL-4B and Cibacron Blue sepharose CL-6B were from Pharmacia (Uppsala, Sweden), and DE-52 was obtained from Whatmann (Kent, UK). Reactive red agarose and heparin sepharose were obtained from Sigma (St Louis, MO), and hydroxylapatite was from BioRad (Hercules, CA). All other chemicals were purchased from Sigma.

Bacterial strains and growth conditions

The *T. thermophilus* strain HB8 was used in all experiments. Microorganisms were grown at 70°C in a medium containing 0.5% (w/v) tryptone, 0.3% (w/v) yeast exract, 0.2% (w/v) NaCl, 0.1% (w/v) glucose, 2 μM $FeCl_3$, 0.2 mM $CaCl_2$ and 1 mM $MgCl_2$. The pH was adjusted to 7.0 with NaOH. Growth was monitored by measuring the turbidity at 600 nm. The bacteria were harvested in the static phase by centrifugation at 6000 *g* for 10 min. Cells were washed twice with 0.9% (w/v) NaCl. The final yield was about 5 g of wet cells per liter of culture medium.

Assay for L-asparaginase

The enzyme activity was measured directly according to the method of Bergmeyer[21] by measuring the ammonia produced from the hydrolysis of L-asparagine with Nessler's reagent. The routine assay by ammonia Nesslerization was performed in a total volume of 1 ml of buffer 50 mM Tris–HCl (Tris-(hydroxymethyl) aminomethane), pH 10.0, containing 10 μmol of L-asparagine and enzyme preparation. The samples were incubated for 20 min at 60°C. The reaction was stopped by the addition of 0.25 ml of 15% trichloroacetic acid (TCA). The protein precipitate was removed by centrifugation and 3.25 ml H_2O added in the supernate. The ammonia liberated was determined colorimetrically with the addition of 0.5 ml Nessler's reagent at 425 nm. L-Asparaginase activity was assayed also indirectly following the formation of NAD^+ in a system where the L-asparaginase reaction was coupled to the reaction of aspartate aminotransferase and malate dehydrogenase.[22] All assays were carried out at 60°C (pH is about 9.2 at 60°C).

The pH values for all buffers were measured at 25°C. The effect of temperature on pH for each buffer was taken into account and all pH values were corrected to L-asparaginase assay temperature to 60°C by the $-dpH/dt$ (unit/degree) coefficient, specific for each buffer.

One international unit (IU) of L-asparaginase activity is defined as the amount of enzyme liberating 1 μmol NH_3 or aspartic acid in 1 min incubated at 60°C under the conditions specified above. Specific activity of L-asparaginase is defined as the units/mg protein.

Glutaminase activity

Glutaminase activity was measured by direct Nesslerization as described above.

Purification of *T. thermophilus* L-asparaginase

T. thermophilus from the stationary phase (50 g) were suspended in buffer A (50 mM Tris–HCl, pH 8.5, 1 mM β-mercaptoethanol and 0.3 mM phenylmethylsulfonylfluoride) (3 ml/g cells). The cells were disrupted by sonic vibration in a oscillator (UP200S, Dr Hielscher) and ultracentrifuged at 105 000 *g* for 1 h.

Solid ammonium sulfate was added to the 105 000 *g* supernatant and the pellet was (20–40% saturation) dissolved in 25 ml buffer A and dialysed overnight at 4°C against the same buffer. The dialysed sample was diluted 1:10 with buffer 50 mM Tris–HCl, pH 8.5, and passed through a DE-52 column (11 × 2.5 cm) equilibrated with the same buffer. After washing the column with 600 ml of buffer 50 mM Tris–HCl, pH 8.5, the bound proteins were eluted with a 500 ml 0–0.2 M NaCl gradient. Fractions were collected and assayed for L-asparaginase activity.

Active fractions were pooled and applied to a phenyl Sepharose CL-4B column (11 × 1.8 cm) equilibrated with buffer 50 mM Tris–HCl, pH 8.5, and 50 mM NaCl. After washing the column with 250 ml of the same buffer, the bound proteins were eluted with 100 ml 20% ethylene glycol and 20–70% ethylene glycol gradient. Fractions were collected and assayed for L-asparaginase activity. Active fractions were pooled and diluted 1:1 with buffer 50 mM Tris–HCl, pH 8.5.

The enzyme solution was applied to a Cibacron Blue Sepharose CL-6B column (10 × 1.6 cm) equilibrated with buffer 50 mM Tris–HCl, pH 8.5, and 25% ethylene glycol. After washing the column with 250 ml of the same buffer, the bound proteins were eluted with a 200 ml 0–0.3 M NaCl gradient. Active fractions were combined and applied on the next column.

Active fractions were diluted 1:3 with buffer 50 mM Tris–HCl, pH 8.5, and applied to a Reactive Red agarose column (7 × 1.4 cm) equilibrated with buffer 50 mM Tris–HCl, pH 8.5, and 50 mM NaCl. The column was washed with 150 ml of the same buffer and the bound proteins were eluted with 100 ml 0.05–

0.8 M NaCl gradient. Active fractions were pooled, dialysed against 5 mM Na_2HPO_4–NaH_2PO_4 pH 8.0 (buffer B) and passed through a hydroxylapatite column (6 × 1.2 cm) equilibrated with buffer B. The enzyme appeared in the flowthrough of the column.

The enzyme solution was applied to a heparin–sepharose column (5.5 × 1.1 cm) equilibrated with buffer B. After washing the column with 100 ml of the same buffer, the bound proteins were eluted with a 50 ml 0–0.5 M NaCl gradient.

Protein determination

Protein is determined by the method of Bradford[23] as modified by Bearden[24] using bovine serum albumin as a standard.

Cell lines and culture conditions

Cell lines used were K-562 (chronic myelogenous leukemia), Raji (Burkitt's lymphoma), SK-N-MC (primitive neuroectodermal tumor), HeLa (human cervical cancer), BT20 and MCF7 (human breast cancers), HT-29 (Human colon cancer), and OAW-42 (human ovarian cancer).

Adherent cells at the logarithmic growth phase were plated (100 μl/ well) in 96-well flat-bottomed microplates at densities of 10 000 cells/well (for SK-N-MC, BT-20, HT-29, MCF-7 and OAW-42 cells), 5000 cells/well (for HeLa cells) and 50 000 cells/well (for K-562 and Raji cells). The microplates containing the cells were preincubated for 24 h to allow cell stabilization prior to addition of agents. Agents at twice the required final concentration in growth medium (100 μl aliquots of each dilution) were applied to triplicate culture wells and microplates were incubated for the appropriate time.[25]

Bioassay

Cytotoxicity was evaluated by an optimized sulforhodamine B (SRB) assay.[26] Absorbance was measured on a spectrophotometric plate reader (Anthos Labtec, Salzberg, Germany) at 492/620 nm wavelength. Results are expressed as percentage of untreated control cells and dose–response curves were plotted.

Results and discussion

Treatment of different tumor breast cell lines with increasing concentrations of L-asparaginase results in appreciable inhibition of the cell growth. The assessment of *in vitro* cytotoxicity was performed by SRB assay 72 h of continuous exposure to *T. thermophilus* asparaginase and Erwinase (Figure 1). The growth inhibition data were expressed as percent of control. The sensitivity of HeLa, SK-N-MC and OAW-42 cells to both asparaginases appeared to be dose dependent, resulting in a significant decrease in viable cells. In HeLa cells this decrease in viability reached almost 70% by both asparaginases when 0.4 IU/ml of enzyme was used. SK-N-MC cells proved to be more sensitive to *T. thermophilus* L-asparaginase than to Erwinase while both enzymes show the same effect on OAW-42 cells.

A similar effect was obtained by *T. thermophilus* L-asparaginase with the other two breast cancer cell lines (MCF7 and BT20) indicating that BT20 are more sensitive to Erwinase (Figure 1). The decrease in viable cells in HT29 by *T. thermophilus* L-asparaginase was around 93% with 0.5 IU/ml of enzyme, while Erwinase at the same concentration (0.5 IU/ml) was less effective and inhibited HT29 cells by 62%.

Comparing the action of the two asparaginases on K562 and Raji cells (Figure 1), we observe that although both enzymes exert large cytotoxicity at 0.5 IU/ml on both cell lines, K562 were found to be less sensitive to Erwinase and Raji were less sensitive in L-asparaginase purified from *T. thermophilus*.

The sensitivity of different cell lines for both L-asparaginases at different times is shown in Figure 2. As we can see, HeLa cells are more sensitive to *T. thermophilus* L-asparaginase than to Erwinase, while in Raji cells we observed the reverse phenomenon. The HT29 are more sensitive to *T. thermophilus* L-asparaginase and at 72 h the survival fraction is close to zero (Figure 2).

L-Asparaginase of *T. thermophilus* was purified to homogeneity by ammonium sulfate precipitation and different chromatographic columns. The enzyme was purified 8840-fold and had a specific activity of 840 IU/mg protein. L-Asparaginase from *T. thermophilus* is a very stable enzyme and even 72 h at 37°C more than 60% of the activity maintained. The enzyme of *T. thermophilus* is a multimeric protein of approximately 200 kDa and a subunit size of 33 kDa.[27] In *T. pyriformis* the purified L-asparaginase exhibits a kinase activity as well and it is autophosphorylated on tyrosine residues. Phosphorylation, or dephosphorylation, of L-asparaginase resulted in a complete loss or activation by more than 10-fold of its catalytic activity, respectively.[28]

The purified enzyme from *T. thermophilus* presents optimal activity at pH 9.2, an isoelectric point of 6.0 and a K_m for L-asparagine of 2.8 mM. The K_m values of L-asparaginases vary from 0.01 to 7 mM. In the literature, L-asparaginases from *S. marcescens*, *Erwinia aroideae* or *T. pyriformis* with K_m values close to that

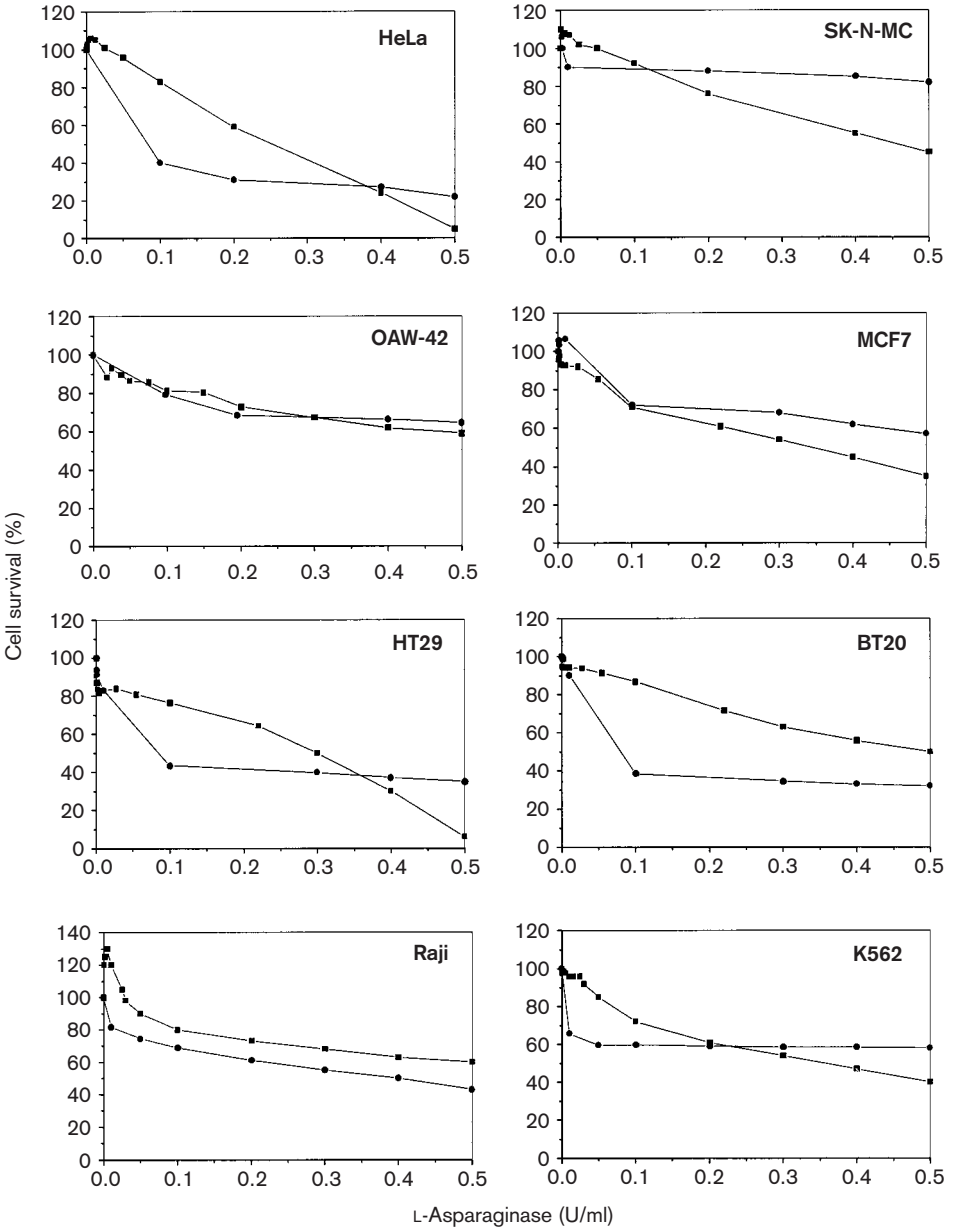

Figure 1. Dose–response curves for SK-N-MC, HeLa, OAW-42, MCF7, HT-29, BT20, Raji and K-562 cells against L-asparaginase from *T. thermophilus* (■) and Erwinase (●) (72 h treatment). Data points represent the average value from a duplicated experiment calculated from at least six replicate wells subtracting background and determined by the SRB microtitration assay.

of *T. thermophilus* L-asparaginase have been reported to possess antitumor activity.[11,29,30] Our purified enzyme does not hydrolyse D-asparagine or L-glutamine as is shown in Table 1. However, L-asparaginase is inhibited when D-asparagine or L-glutamine is included in the assay mixture. The fact that L-asparaginase of *T. thermophilus* is free of glutaminase activity makes it more advantageous for future clinical studies, since one of the reported side effects of L-asparaginases is the hydrolysis of L-glutamine and its depletion from the serum.[16,31]

We had reported that the breast cell lines used in this investigation do not contain L-asparagine synthetase activity.[11] Therefore, the selective growth inhibition by L-asparaginase of breast cancer cells and possibly of the other tested cell lines could be related to the absence of intracellular L-asparagine synthetase activity in these cells.

Our results show that L-asparaginase purified from *T. thermophilus* presents antiproliferative activity in different cell lines grown *in vitro*. It is obvious that more general conclusion will have to be made by using higher concentrations of L-asparaginase. The fact that *T. thermophilus* enzyme is active in these cell lines indicates that efforts will be made to investigate further other target cells in combination with other antiproliferative drugs as well.

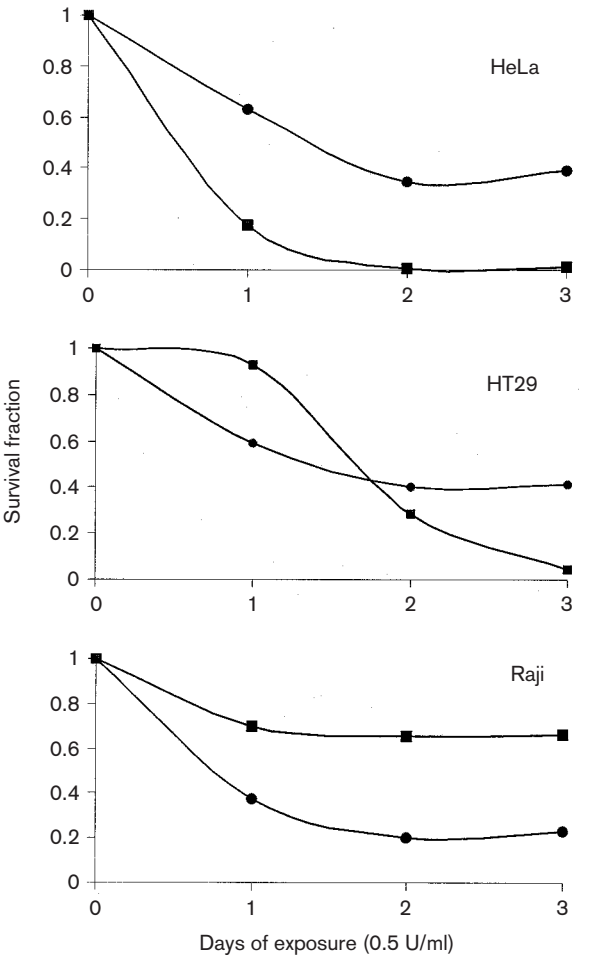

Figure 2. Survival fractions of HeLa, HT29 and Raji exposed to L-asparaginase from *T. thermophilus* (■) or Erwinase (●) for 24, 48 or 72 h. Data points represent the average value from a duplicated experiment calculated from three replicate wells subtracting background and were estimated by the SRB assay.

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Table 1. Hydrolysis of L-asparagine, D-asparagine and L-glutamine by L-asparaginase from *T. thermophilus*, and their interference on L-asparaginase activity

Substrate	Concentration (mM)	Relative activity of L-asparaginase (%)	Inhibition of L-asparaginase[a] (%)
L-Asparagine	10	100	–
D-Asparagine	10	0	33
D-Asparagine	20	0	53
L-Glutamine	10	0	7, 5
L-Glutamine	20	0	15

100% relative activity was defined the hydrolysis of 10 mM L-asparagine by L-asparaginase at the specified conditions.
[a]When the inhibition of L-asparaginase was measured the assay mixture contained 10 mM L-asparagine and the above indicated concentration of D-asparagine or L-glutamine.

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