Comparative Study of L-asparaginase Activity in Different Bacterial Species

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Summary

42 bacterial species including nine isolates from soil of Iran that were previously isolated and characterized as L-asparaginase producing microorganisms and 33 species from Razi Institute Type Culture Collection were examined to investigate their L-asparaginase activity. All of them were cultured in flasks containing M9 medium with L-asparagine as the only nitrogen source. Their growth, presence of L-asparaginase and its quantity were studied. According to the results some of the bacterial species did not grow in the medium, some grew but without any L-asparaginase activity and some with enzyme activity in different quantity. Among them three different bacterial species including a *Pseudomonas flourscence* and a *Bacillus pomilus* isolated from soil of Iran, and a *Providencia stuartti* from Razi Institute Type Culture Collection had higher activity up to 3 times the level of known species such as *Erwinia carotovora* and *Escherichia coli*. It is noteworthy that there are no reports describing the existence of L-asparaginase in *Providencia* and *Bacillus pomilus* today.

Key words: L-asparaginase, L-glutaminase, *Pseudomonas flourscence, Bacillus pomilus*, *Providencia stuartti*, antileukemic enzymes

Introduction

L-asparaginase or L-asparagine amidohydrolase (EC 3.5.1.1) is an enzyme that converts L-asparagine to L-aspartic acid and ammonia. After the assess that antileukemic activity of guinea pig serum was due to its L-asparaginase activity (Broom 1996) and isolation of this enzyme from *Escherichia coli* that had similar activity (Roberts *et al* 1996) a considerable attention was attracted to this area of research. The clinical action of this enzyme is attributed to the reduction of circulating L-asparagine. Since certain neoplastic cells are dependent on extracellular

supplies of this amino acid, they are selectively killed by L-asparagine deprivation (Haley *et al* 1961). In order to administration of the enzyme that extracted from *E.coli* produce immune reactions in body. Some studies had been done to find new sources of this enzyme with anticancerous activity that could differ immunologically from *E.coli* enzyme. These studies resulted in introduction of *Erwinia* L-asparaginase which did not have any immunological cross-reactivity with *E.coli* one's and so could provide an alternative therapy for patients who have become hypersensitive to one of these enzyme (Cheristopher 1994, Moola *et al* 1994). The glutaminase asparaginase from *Pseudomonas* 7A (PGA) has shown significant antitumor activity in mice (Jacob *et al* 1997) especially when combined with glutamine antimetabolites and recently has shown promise as a possible therapeutic agent for the treatment of retroviral diseases (Roberts & McGregor 1991). Some vaccines have been used for immunotherapy of cancers such as lymphoblastic leukemia and breast cancer and are suggested that the therapeutic effect of them seems to have some relation to their L-asparaginase activity (Horobagyi *et al* 1980). It is also suggested that this enzyme has immunosupressive effects on immune system (Berenbun 1970, Durden *et al* 1980) and inhibits lymphocyte proliferation *in vitro* (Chuang *et al* 1990).

Recently a new plate assay method has been introduced for rapid screening of the microorganisms that produce the enzyme (Gulati *et al* 1997). The activity of this enzyme in limited number of microorganisms was compared but more studies are underway. In this study a wide variety of bacterial species including some known sources of this enzyme, together with some new bacterial species were studied.

Material and Methods

Chemicals. L-asparaginase (Sigma), glutamate dehydrogenase, NADH, ADP (Boehringer Manneheim), L-asparagine monohydrate and L-glutamine (Fluka) were obtained. All other chemicals were purchased from commercial suppliers.

Bacterial strains. All of bacteria with RTCC number were provided from Razi Institute Type Culture Collection. The remaining were isolated by Aghaipour (unpublished data) from soil of Iran.

Growth of bacteria. Cells were grown in modified M9 medium which formulated as:

$Na_2HPO_4,12H_2O$ 12g/l
KH_2PO_4 ..... 3g/l
NaCl ..... 0.5 g/l

L-asparagine monohydrate..... 5 g/l
Glucose..... 2 g/l
$MgSO_4,7H_2O$..... 0.5 g/l
$CaCl_2,2H_2O$.....15 mg/l

The pH was adjusted to 7.0 and autoclaved 15 min at 120C. The bacteria were cultured in 1 liter flasks each containing 250ml medium with shacking in a rotary shaker incubator (100 rpm).

Cell harvesting. Cells were harvested at different time of growth phase by centrifugation at 10,000*g* for 10 min. After two washings with 100mM tris hydrocloride (pH 8.4) containing 1M KCl, the cells were suspended in 10% of its initial volume of 10mM tris hydrocloride (pH 8.4) containing 10mM mercaptoethanol.

Cell lysis. Cells were broken by two methods. The first method was sonication for a total time of 5 min and centrifugation for 20 min at 40,000*g*. The second method was vortexing 1ml of cell suspension in the presence of 40µl of toluene and subsequent centrifugation.

L-asparaginase and L-glutaminase activity assay. L-asparaginase activity assay was performed with direct nesslerization (Bregmeyer 1974) or coupled enzymatic method using NADH and glutamate dehydrogenase (Bregmeyer 1983) at 37C. L-glutaminase activity was measured as described by Wade (1971). The only modification was replacing tris buffer for borate.

Protein assay. Protein determination was done by Bradford (1974) method.

Unit of enzymatic activity. The amount of enzyme that converts 1µMol of L-asparagine to aspartic acid and ammonia per min was measured.

Results and Discussion

In this study a wide variety of bacterial species were studied to monitor their growth and L-asparaginase activity. Modified M9 medium was used which contained L-asparaginase as the only nitrogen source. There are at least three metabolic pathways for asparagine degradation, which two of them are known in details. The first is via asparaginase catalysed amide hydrolysis to form aspartate and ammonia. This is the main pathway in many cells that could use asparagine. A second pathway for degradation of asparagine involves transamination of asparagine followed by amide hydrolysis and the two enzymes shown to be involved are asparagine

aminotransferase and ω-amidase. It is thought there are other pathways for degradation of asparagine that is not completely known (Moraga *et al* 1989).

In this study the asparaginase activity of 42 bacterial species were studied and compared with each other. Previous studies have been suggested both constitutive and inductive mechanisms for expression and synthesis of this enzyme in different bacteria (Svobodova & Strbanova-Necinova 1973) and M9 medium could be used for expression and synthesis of asparaginase in both of these mechanisms (Gulati *et al* 1997). Asparaginase activity assay was performed on whole cells, sonicated cell extract and organic solvent disrupted cell extract and compared with each other. According to obtained results these bacteria could be classified in three groups. Some could not grow on M9 medium at all, like *Lactobacillus* species. Some grew, *Micrococcus* species, but did not have any L-asparaginase activity and the rest had different levels of enzymatic activities (Table 1).

All of the studied *Lactobacillus* spp., *Eikenella Corrodens*, *Flavimonase oryzihabitans*, *Gluconobacter oxidans*, *Moraxella bovis* and *Providencia alcalifaciens* strains could not grow in this medium. It could be said that the growth failure could possibly arisen from the deficiencies in some or all enzymatic pathways i.e. asparaginase pathway or transaminase pathway, that are responsible for utilization of nitrogen from asparagine (Moraga *et al* 1989, Huerta-Zepeta *et al* 1997). The other possibility could be the necessity of these microorganisms to some other ingredients in addition to components of this medium to grow. *Micrococcus luteus* and *E. coli* strains in spite of their good growth had no enzymatic activity. It seems that these microorganisms used other enzymatic pathways such as transaminase pathway rather than L-asparaginase for providing nitrogen for their growth from asparagine and asparaginase pathway does not exist in these bacteria.

The other bacteria had asparaginase activity in different amounts. These included *Streptomyces* spp., *Bacillus* spp., *P.flourscence, Klebsiella pneumonia, Citrobacter freundii, Aearomonas* spp., *Edwardsiella tarda, Erwinia carotovora* strains, *E.coli* strains, *Hafnia alvei, Moraxella bovis, Nocardia* spp., *Providencia rettgeri* and *Providencia stuartti* which had asparaginase activity but its amount was very different between them. Nine of these bacteria were previously isolated from Iran and the remaining was from Razi Institute Type Culture Collection (Table 1). In all of above bacteria, asparaginase activity of cell lysates were 80-90% of whole cell except for *P.flourscence* which activity of cell lysate was about two time's of whole cell. These data support the general belief that activity of most microbial

asparaginase are fully expressed in the intact organism and suggest that these enzymes are generally located in the periplasm like antitumour asparaginase from *E.coli* (Wade *et al* 1971).

Table 1. *Comparison the L-asparaginase activity between different cultured bacterial species in M9 medium*

Bacterial Species	RTCC Number	L-asparaginase activity	
		Unit/ml broth	Unit/mg wet biomass
Streptomyces sp. (103)	*	0.058	0.009
Streptomyces sp. (104)	*	0.109	0.010
Bacillus pomilus (105)	*	0.236	0.193
Bacillus sp. (106)	*	0.128	0.018
Pseudomonas flourscence (107)	*	0.353	0.086
Bacillus sp. (108)	*	0.186	0.019
Bacillus sp. (110)	*	0.033	0.021
Klebsiella pneumonia (116)	*	0.070	0.013
Citrobacter freundii (117)	*	0.064	0.019
Aeromonas hydrophila	1027	0.081	0.019
Aeromonas sorbia	1028	0.079	0.012
Aeromonas hydrophila	1039	0.118	0.027
Aeromonas hydrophila	1030	0.067	0.013
Aeromonas hydrophila	1031	0.077	0.013
Edwardsiella tarda	1143	0.036	0.011
Eikenella corrodens	1144	NG	NG
Erwinia carotovora	1152	0.116	0.033
Erwinia carorovora	1153	0.112	0.032
Erwinia carotovora	1154	0.091	0.031
Escherichia coli O86	1163	0.023	0.007
Escherichia coli O125	1173	0.000	0.000
Escherichia coli	1199	0.082	0.038
Escherichia coli	1201	0101	0.039
Flavimonas oryzihabitans	1217	NG	NG
Gluconobacter oxidans	1225	NG	NG
Hafnia alvei	1235	0.050	0.019
Lactobacillus casei	1265	NG	NG
Lactobacillus delbruckii	1269	NG	NG
Lactobacillus fermentum	1270	NG	NG
Lactobacillus leichmannii	1272	NG	NG
Lactobacillus plantarum	1273	NG	NG
Moraxella bovis	1340	NG	NG
Nocardia asteroids	1389	0.016	0.008
Nocardia brasiliens	1391	0.041	0.006
Nocardia brevicatesa	1392	0.021	0.025
Nocardia carnae	1393	0.049	0.026
Providencia alcalifaciens	1468	NG	NG
Providencia alcalifaciens	1470	NG	NG
Providencia stuartti	2112	0.291	0.042
Providencia rettgeri	2119	0.082	0.019
Micrococcus luteus	1834	0.000	0.000
Micrococcus luteus	1836	0.000	0.000

* : These bacterial species have been isolated from soil of Iran
RTCC : Razi Institute Type Culture Collection
NG : No Growth

The small decrease in cell lysates activity could be due to release of cellular protease or physical denaturation during cell disruption. In *P.flourscence* increase of activity could be because of release some intracellular asparaginase. Among isolates from Iran, there were two microorganisms with good L-asparaginase activity that had been characterized as *P.flourscence* and *B.pomilus*. There have been no reports on existance of L-asparaginase in *B.pomilus* todate, and this is the first report on these bacteria. Among Razi Institute Type Culture Collection studied species, it was found a *P.stuartti* with good L-asparaginase potency. It is noteworthy that there is no report on the existence of L-asparaginase in *Providencia* genus today. Among 4 species of *Providencia* that we studied, two were not capable of growth in this medium and one had only a slight growth, but only this one had both good growth and contained a considerable amount of enzyme. It is seems that although asparaginase pathway had possibly weak or no role in nitrogen utilization in other studied *Providencia* spp., but in *P.stuartti* a strong pathway is present.

Table 2. *Comparison the existence and amount of L-glutaminase related to L-asparaginase activity*

Bacterial species	L-asparaginase activity	L-glutaminase Activity
Pseudomonas flourscence	100%	100%
Bacillus pomilus	100%	37%
Providencia stuartti	100%	<1%

As mentioned previously L-asparaginase is an antileukemic agent with some side effects including immunosupression. It has been mentioned that most of these side effects are due to glutaminase activity of this enzyme. The enzyme with less glutaminase activity was reported to have fewer side effects (Distasio *et al* 1982). Some investigators had used glutamine anti metabolites with this enzyme during administration to reduce its side effects (Jacob *et al* 1997). In present study glutaminase activity of the above three microorganisms are investigated (Table 2). *P.stuartti* showed no glutaminase activity but *B.pomilus* and *P.flourscence* had 37% and 100% glutaminase activity regarding their asparaginase activity, respectively. This is in agreement with the reported results for *P.flourscence* that says it has a asparaginase with glutaminase activity or asparaginase glutaminase.

The above results showed that the characteristic of metabolic pathways for nitrogen utilization were different in the microorganisms under study and L-asparaginase from *P.stuartti* could have antileukemic activity with less side effects.

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