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The Isolation of L-Asparaginase and L-Serine Dehydratase from Bacteria*

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Introduction

Kidd (1953) first observed that certain transplanted lymphomas of mice and rats were strongly suppressed by treatment with guinea pig serum, and Broome (1961) provided evidence that L-asparaginase in the serum is the antitumoral factor. The finding by Mashburn and Wriston (1964) that L-asparaginase derived from Escherichia coli has antitumor activity similar to that of guinea pig serum opened up the possibility of large scale production of the enzyme for ultimate clinical trial. Thus, a large amount of purified enzyme has been supplied and employed in the clinical tests for acute leukemia and other malignant neoplasms in man by Hill, Roberts, Loeb, Khan, Maclellan, Hill (1967), Hill, Loeb, Maclellan, Khan, Roberts, Schields, Hill (1969) and Oetgen, Old, Boyse, Campbell, Philips, Clarkson, Tallal, Leeeper, Schwartz, Kim (1967). Khan and Hill (1969) showed that the administration of such an enzyme protein for a long duration produces the corresponding antibody in the living bodies, and the antibody causes and anaphylactic schok or neutralization of the drug effect. Therefore, the discovery of a new L-asparaginase immunologically different from that of E.coli has been greatly desired. Thus, in addition to E.coli some reports have been published on the production of tumor inhibitory L-asparaginase from Aspergillus terreus by DeAngeli, Pochiari, Tonolo, Zurita, Ciaranfi, Perin (1970), from Erwinia aroideae by Peterson, Ciegler(1969), from E.carotovora by Wade, Elsworth, Herbert, Keppie, Sargeant (1969), from Mycobacterium tuberculosis by Jayaram, Ramakrishnan, Vaidyanathan (1968), from Serratia marcescens by Heinemann, Howard, Palocz (1970), and

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from Proteus vulgaris by Tosa, Sano, Yamato, Nakamura, Ando, Chibata (1971),. At the other hand, in recent years Regan, Vodopick, Takeda, Lee, Faulcon (1969) showed that leukocytes from chronic granulocytic leukemia apparently require serine for growth. It has been suggested that L-serine dehydratase might be useful in leukemia therapy.

In the present paper twenty nine strains of bacteria and five strains of fungi were tested for L-asparaginase and L-serine dehydratase activity, and partially purified L-asparaginase from P.vulgaris was measured for the effect of synthesis of DNA, RNA and protein on acute leukemia cells.

Materials and Methods

Bacterial and Fungal Strains: The bacteria and fungi marked with QMB were obtained from Quatermaster Research and Development Center U.S.Army, Natick, Mass. Culture Collection. E.coli N 300, E. coli B,E.coli B_1 and E.coli W were obtained from Michrobiology department, Harvard University. E.Coli K_{12}687 and E.coli K_{12}AB 735 were obtained from Microbiology department, California University. E.coli C 600/5 was obtained from Microbiology Department, Sussex University. P.vulgaris A-232 and other strains of bacteria without code number were obtained from Institute of Hıfzısıhha, Ankara,

Media and Culture Method: To screen for L-asparaginase producing bacteria, a medium developed by Bilimoria (1969), containing 0.1 % pepton (Difco), 0.6 % beef extract(Difco), 0.33% KH_2PO_4 and 0.1 % L-asparagine was used. Aspergillus cultures were grown on a medium of Deangeli, Pochiari, Tanola, Zurita, Ciaranfi and Perin (1970), containing 5 % pepton(Difco), 1 % beef extract(Difco) and 5 % mannitol. Media used for bacteria were adjusted to pH 7.0 and for fungi to pH 4.5 befor sterilization at 121°C of presure for 20 min. The media were distributed in 50 ml amounts to 250 ml shaking flasks, sterilized, and inoculated with a loopful of bacteria. Culture was carried out for 24 hours at 37°C on a reciprocating shaker (New Brunsvick Scientific Co) at 175 rev/min. Five days old slant cultures of Aspergillus were suspended with 10 ml of distilled water and 1 ml of this suspention was used for inoculation. The strains of Aspergillus were grown at 30°C for 48 hours on a reciprocating shaker at 175 rev/min. Production was collected by centrifugation and the cell paste was then frozen and kept at-20°C.

L-Asparaginase Assay: L-Asparaginase activities were measured by the procedure described by Peterson and Ciegler (1969) with some modifications Frozen cells were suspended with distilled water. A 0.1 ml sample of cell suspention, 0.9 ml of 0.1 M sodium borate buffer (pH 8.5),

and 1 ml of 0.04 M L-asparagine solution were combined and incubated for 10 min at 37 C. The reaction was stoped by the addition of 0.5 ml of 15 % (W/V) trichloracetic acid. After centrifugation, a 0.5 ml portion of supernatant fluid was diluted to 4 ml with distilled water and treated with 1.0 ml of Nessler's reagent. The colour reaction was allowed to proceed for 15 min. The yellow colour was read at 450 nm in a Bausch-Lomb spectrophotometer. The OD was then compared to a standard curve prepared from solution of ammonium chloride as the ammonia source. One international unit (IU) of L-asparaginase is that amount of enzyme which liberates 1 micromole of ammonia in 1 min at 37 C. The remainder of the cell suspention was dried and the dry weight determined.

L-Serin Dehydratase Assay: L-Serine dehydratase activities were measured by the method of Nelson, Peterson, Ciegler (1973). The only difference of this method from that of L-asparaginase assay is the buffer used in the assay as 0.01 M.

Partial Purification of L-asparaginase: All of the steps described below were carried out at 4 C. Frozen cells were thawed and suspended with 100 ml of 0.05 M phosphate buffer (pH 7.0) per 10 g cell paste, cooled in ice and sonicated in a Artek Model 150 Sonicator for 15 min. Cell free extract was collected by centrifugation (30 min at 30000xg) and treated with 0.05 volumes of 1.0 M $MnCl_2$ to partially remove nucleic acid. After the $MnCl_2$ was added, the suspention was stirred for 1 hr, allowed to stand for 15 min, and then centrifuged to remove precipitate. Solid $(NH_4)_2SO_4$ was added to the supernatant fluid to a concentration of 25 % saturation. After stirring for 30 min, the precipitate was collected by centrifugation at 17.000 rpm for 20 min. The $(NH_4)_2SO_4$ concentration was raised to 40 % and then 80 % saturation. The precipitates of each obtained was centrifuged as before. The $(NH_4)_2SO_4$ precipitated fractions were then dialized against 0.05 M phosphate (pH 7.0) and the dializate was kept at –20°C.

Anti Leukemic Test: The anti leukemic action of L-asparaginase was tested against granulocytic leukemia cells. Leukemic blood was obtained from the Children Hospital, Hacettepe University. A 0.25 ml of heparinized blood was incubated in 5 ml of Dulbecco's modified Eagle medium with 10 per cent dialyzed (against 30 volumes of normal saline, with three changes at 4°C for 48 hours) calf serum at 37°C. A 1 IU of L-asparaginase was added to the assay tube. Deoxyribonuclei acid (DNA) and Ribonucleic acid (RNA) synthesis was measured by incorporation of tritiated thymidine (1 uc/ml) and tritiated uridine (1 uc/ml) into a fraction insoluble in trichloracetic acid by using filter disc method of

Bollum (1968). Protein synthesis was measured by the procedure described by Mans and Novelli (1961), using tritiated methionine (1 uc/ml). **Protein Determination:** Protein was determined by the method of Lowry, Rosebrough, Farr and Randall (1951).

Results

Twenty nine strains of bacteria and five strains of fungi were screened for L-asparaginase and L-serine dehydratase production. The results of these are summarized in Table I. Proteus vulgaris A-232 was found to contain high levels of these enzymes. Of the other bacteria tested E. coli W, Serratia marcescens QMB 466 were potent sources of L-serin dehydratase. Clinically isolated strain of E.coli was found to synthesize remarkable quantities of L-asparaginase. Proteus vulgaris was selected for further studies because of high productivity on the production of both enzymes.

During the $(NH_4)_2SO_4$ purification steps the highest L-asparaginase activity was found at 80 % saturation and after sonication L-serin dehydratase activity was lost. In addition, when relevant substrates were present in the growth medium, both of the enzymes induced (Table II). Partially purified L-asparaginase which was obtained from P.vulgaris A-232 inhibited DNA, RNA and protein synthesis of acute leukemia cells by 10 %, 3 %, 9 % respectively (Table III).

Conclusion

It has been shown that P.vulgaris A-232 produced both of the enzymes in high levels, this may give an advantage to produce the enzymes from one organism. Nelson, Peterson and Ciegler(1973) reported high yield of L-serine dehydratase from P.vulgaris NRRl B-123, but didn't measure L-asparaginase activity. In addition, an other strain of P.vulgaris was found to have high L-asparaginase activity. by Tosa, Sano, Yamato, Nakamura, Ando and Chibata (1968).

When the appropriate substrates added to the growth medium, the production of enzymes are increased. This suggested that the enzymes are inducible or substrates act as a nitrogen source.

Loosing the activity of L-serin dehydratase by sonication, might be from degradation of molecule during this procedure.

The inhibition of DNA, RNA and protein synthesis of acute leukemia cells by L-asparaginase from P.vulgaris A-232, shows that it has an anti tumoral activity, and possibly willi increase this property with further purification.

TABLE I

Production of Asparaginase and Serine Dehydratase by Bacteria and Fungi

Organism	L-asparaginase i.u./mg dry weight	L-serine dehydratase i.u./mg dry weight	mg dry weight /ml medium
E.coli B	0.462	0.035	0.70
E.coli W	0.179	0.271	0.46
E.coli N 300	0.086	0.000	0.60
E.coli C 600/5	0.106	0.094	0.76
E.coli B1	0.083	.041	0.60
E.coli K12687	0.123	0.000	0.60
E.coli K12AB 735	0.104	0.000	0.76
E.coli*	1.030	0.083	0.60
Serratia marcescens QMB 466	0.040	0.126	0.34
Bacillus suptilis QMB 1611	0.087	0.000	0.040
Bacillus cereus QMB 1565	0.035	0.000	0.85
Bacillus megaterium QMB 1605	0.000	0.100	0.60
Bacillus circulans QMB 1569	0.000	0.000	–
Bacillus alvei QMB 1593	0.000	0.000	–
Bacillus badius QMB 1595	0.000	0.000	–
Bacillus brevis QMB 1570	0.000	0.000	–
Bacillus licheniformis QMB 1603	0.000	0.000	–
Bacillus sphericus QMB 1609	0.000	0.000	–
Alcaligenes fecalis QMB 1483	0.000	0.000	–
Cellulomonas uda QMB 1534	0.000	0.000	–
Cellvibrio vulgaris QMB 2	0.000	0.000	–
Pseudomonas ovalis	0.000	0.035	0.70
Pseudomonas aeroginosa	0.063	0.037	0.90
Proteus vulgaris A-232	0.648	0.170	0.96
Proteus vulgaris	0.403	0.045	0.75
Proteus vulgaris	0.475	0.015	0.80
Proteus mirabilis	0.079	0.015	0.76
Micrococcus flavus	0.000	0.000	–
Aerobacter aerogenes	0.468	0.000	0.36
Aspergillus foetidus QM 328	0.000	0.000	–
Aspergillus carbonarius QM 331	0.000	0.000	–
Aspergillus niger QM 1273	0.000	0.000	–
Aspergillus phoenicus QM 329	0.000	0.000	–
Aspergillus nidulans QM 1985	0.000	0.000	–

* Clinically isolated strain.

TABLE II

Effect of the Substrates on the Production of Enzymes by P.vulgaris

Experiment*	L-Asparaginase IU/ml medium	L-Serine dehydratase IU/ml medium
1. Control	0.540	0.180
2. + L-asparagine	0.800	0.150
3. + L-serine	0.590	0.210
4. + L-serine and L-asparagine	0.700	0.250

* In all experiments shown in the table, same media (0.1 % pepton, 0.6 % beef extract, 0.33 % KH2PO4) was used, and amino acids were added to this medium in 0.1 % concentrations except control.

TABLE III

Effect of L-asparaginase on the Synthesis of DNA,RNA and Protein on acute leukemia Cells.

	Incorporation (%)		
	H3-Thymidine	H3-Uridine	H3-Methionin
Comlete medium	100	100	100
— L-asparagine	80	77	76
+ L-asparaginase	90.2	97	90.8

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Summary

Twenty nine strains of bacteria and five strains of fungi were screened for L-asparaginase and L-serine dehydratase activity. Of these, one strain of P.vulgaris was found to produce high levels of both enzymes. E.coli W and Serratia marcescens QMB 466 were shown to produce L-serin dehydratase in remarkable quantities. Partially purified L-asparaginase which was extracted from P. vulgaris A-232 by sonication, was found to inhibit DNA, RNA and protein synthesis of acute leukemia cells, by 10,3,9, percent recpectively. In addition when relevant substrates were present in the growth medium, both of the enzymes could be induced.

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