SUMMARY

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L-Asparaginase activity was detected in V.cholerae O1 and non-O1 (NAG Vibrio) strains, but L-asparaginase was absent in the strains of other Vibrio species, such as V.parahaemolyticus, V.anguillarum, V.alginolyticus, V.vulnificus, and V.tyrogenes.

Several V.cholerae strains were isolated and characterized by the conventional biochemical and serological tests, and also with the detergents induced agglutination tests by using (flagellar) H-antiserum, and V.cholerae non-O1 antisera.

Bacterial strains were grown in different media for the intracellular and extracellular L-asparaginase formation. The optimum conditions were established for the bacterial enzyme production. The V.cholerae strains produced only intracellular L-asparaginase.

A most suitable inducible medium (i.e., TAYG medium) was composed. The toxigenicity of the V.cholerae strains were determined in the RIL experiment.

The hypertoxigenic strains of V.cholerae showed significantly higher enzyme activity ($p < 0.001$) independent of their biotype and serotype, than those of less or non-toxin producers. The amount of the enzyme was relatively more in strains of V.cholerae O1 than non-O1, and in those of biotype classical than ElTor.

Four consecutive passages through rabbit ileal loops (RIL) enhanced the enzyme as well as enterotoxic activities of hypertoxigenic and less toxigenic strains, but not of the non-toxic ones. The enhanced enzyme production was significantly higher ($p < 0.001$) in highly toxigenic strain (569B) than that in mildly toxigenic ($V63P^-$) and non-toxigenic strains ($CRCV^-$).

The addition of exogenous 3 mM cAMP to the inducible medium increased the enzyme production of non-toxigenic strain upto approximate 4-fold ($p < 0.001$), whereas no such in mildly toxigenic and highly toxigenic strains. The addition of 0.5% glucose exogenously in the inducible medium greatly diminished the enzyme production (about 10-fold) of all the strains.

The addition of 2 mM EDTA and 2 mM glutathione in combination could retain the enzyme activity of highly toxigenic strain much better at $0^{o}C$ and $4^{o}C$ for 180 days (96% retention) and 32 days (81% retention), respectively, than EDTA and glutathione separately. In mildly toxigenic strain, the retention of enzyme activity by the above stabilizers in combination or separately was very poor incomparison with highly toxigenic strain, whereas in non-toxigenic strain the enzyme was not at all stable even in the presence of the used stabilizers.

It was established that for the enzyme production of the 2 different toxigenic strains of V.cholerae, yeast extract and tryptone were most suitable than peptone, malt-extract, beef-extract and corn-steep liquor in the media. Among the inorganic salts tested, the K_2HPO_4 and KH_2PO_4 showed some moderate effect, and the salts of Na^+ were mostly inhibitory to the enzyme formation.

A constant growth culture pH 7.4 was better for the bacterial enzyme biosynthesis, than that of the studied other growth culture pH. In this regard, the synergestic role of 0.25 M potassium phosphate buffer (pH 7.4) was beneficial, instead of 0.05 M Tris-HCl buffer (pH 7.4) and 0.25 M sodium phosphate buffer (pH 7.4).

The adopted re-induction and starvation procedures caused no efficacious contribution in enzyme production of the two different toxigenic V.cholerae strains.

Lactic acid (0.4%) addition to the modified inducible medium (i.e., in TAYG medium-5) caused significant inhibitions (1.4 fold, $p < 0.001$) of enzyme production, as well as 0.6% L-glutamine when added to the medium unable to enhance the enzyme yield of the highly toxigenic strain (569B) and mildly toxigenic strain ($V63P^-$).

More L-asparaginase (1.14 to,es) was produced by the strains 569B and $V63P^-$ when they were grwon only in (0.18%) L-cysteineated medium (i.e., in TAYG medium-6), than in the acysteinated TAYG medium-5. However, this was more pronounced ($p < 0.001$) in the strain 569B but in less ($p < 0.01$) in the strain $V63P^-$.

The so produced enzyme from strain 569B (grown in TAYG medium-6) was less-inhibited by 0.002 M p-OHMB, yet unaffected with the addition of glutathione,

EDTA, DTT and PMSF. The enzyme of the strain was also safe to restore in the present of 0.002 M EDTA only within buffer.

Thermal-inactivation of the enzyme of each strain (grown separately in TAYG and TAYG medium-6) was different. For incubation of each enzyme preparation at 56°C for 10 min, 82% inactivation was observed in the strain V63P⁻; whereas in that case 55% inactivation was observed in strain 569B. While in the same condition, the 15% and 27% less-inactivation (i.e., 70% and 40% inactivation) was noticed respectively for the enzyme of these strains V63P⁻ and 569B when grown in L-cysteine supplemented medium.

L-Asparaginase activity of the sub-cellular fractions of two different toxigenic strains of Vibrio cholerae, e.g., 569B (highly toxigenic) and V63P⁻ (mildly toxigenic) was compared. After the sucrose, EDTA and lysozyme treatment to the cells, followed by sonication and centrifugations to achieve the sub-cellular extracts (lyzates), L-asparaginase activity was detected in both periplasm and cytoplasm extracts of both strains.

L-Asparaginase activity was in the cell-free supernatant (CFSP), periplasm (PP) and cytoplasm (CP) preparations after 35-55% $(NH_4)_2SO_4$ saturated fractionation of each extract of the respective strains. The enzyme activity of the 6 preparations was abolished due to either chilled ethanol (0.5 vol.) or chilled acetone (0.28 vol.) fractionations. $MnCl_2$ (20 mM) and NaCl (150 mM) vividly inhibited the enzyme activity, while KCl (600 mM) retained the same of all. L-Asparaginase of the 3 preparations of strain 569B exhibited more relative velocity at pH 7.4 and in 37°C, less-inactivated in 56°C for 10 min, and by p-OHMB (2 mM); showed higher substrate catalytic efficacy (with 4 mM L-asparagine) than that of all the preparations of strain V63P⁻. No glutaminase activity was found in both the strains.

The enzyme L-asparaginase was purified from cell-free supernatant of V.cholerae strain 569B. Purified enzyme was obtained by $(NH_4)_2SO_4$ fractionation, followed by chromatography on column of DEAE-cellulose, Sephadex G-100, Sephadex G-200, and DEAE-Sephadex, successively. An overall purification of 242-fold, with a yield of 5.53% was obtained. The specific activity of the purified preparation was 88.848 IU/mg protein.

The molecular weight (MW) of the enzyme was determined by gel filtration on Sephadex G-200. An approximate MW of 116,000 ± 4,000 was calculated.

The enzyme appears to be homogeneous, as the purified enzyme preparation migrated as single band in native polyacrylamide gel electrophoresis. Homogeneity of the purified enzyme was also judged by finding symmetrical elution curve, and constant specific activity throughout the peak in DEAE-Sephadex column chromatography, and during the determination of MW of the enzyme in Sephadex G-200 gel filtration.

The sub-unit molecular weight of the enzyme was approximate 29,000 as determined by performing SDS-polyacrylamide gel electrophoresis.

In the nesslerization method the K_m of the enzyme for L-asparagine was 1.42×10^{-4} M at the optimum pH 7.6 and 37 °C. By the hydroxylaminolysis method, the K_m of the enzyme was 1.35×10^{-4} M.

D-Asparagine and DL-asparagine were hydrolyzed at 12% and 76%, respectively, of the rate found with the L-isomer; but L-glutamine was not hydrolyzed at all by L-asparaginase.

The suckling mouse test and the rabbit skin permeability test were performed to assess the probability of presence of endotoxin in the different folds purified enzyme preparations. No untoward results were found.

V.cholerae-L-asparaginase caused inhibition (direct-lysis) to Dalton's lymphosarcoma (DL), and Ascites fibrosarcoma (AFS) tumor cells, during the in vitro studies, where the mean of viable cell count difference at 18 h or the experiments was 0.22×10^6, and at 24 h of the experiments was 0.18×10^6.

Purified L-asparaginase was mostly anti-tumoric when administered on the day of tumor implantation in mice. In the DL-tumor induced mice the enzyme caused an increase in mean survival time for 14 days, or delayed tumor appearance for 13 days, and sometimes about 50% cases of complete remission. The changes in the LD_{50} values of the experimental mice were also significant. The almost same effects were noted for the enzyme administration to the AFS-tumor induced mice.

The enzyme was cleared from the plasma of normal mice with a half-life of approximate 4 h. In the Swiss mice induced with the DL-tumor, the half-life of the administered enzyme was approximate 18 h, whereas in the mice induced with the AFS-tumor it was 15 h. More or less, the same results were noted in the cases of E.coli-L-asparaginase (EC-2) treatment.

The surface ultrastructure and rosetting properties of splenic lymphocytes of the Swiss mice were studied under scanning electron microscope following transplantation of Dalton's lymphoma (DL) and Ascites fibrosarcoma (AFS) tumor cells and administration of V. cholerae-L-asparaginase, or E.coli-L-asparaginase. The study revealed that there was no significant stimulatory or inhibitory effects of V.cholerae-L-asparaginase on the lymphocyte blastogenesis.

The immunological cross-reactivity of the V.cholerae-L-asparaginase with antibodies to E.coli-L-asparaginase (EC-2) was tested with a high-potency rabbit antiserum, by double-diffusion in agar. To clarify cross-reactivity of V.cholerae-L-asparaginase preparation with the anti-EC, different levels of antigen-antibody systems were tested, but no cross-reaction was detected in any instance, indicating that L-asparaginase produced by the V.cholerae was immunologically different from that of E.coli.

Therefore, the V.cholerae-L-asparaginase could possibly become a new useful antitumor drug.