STUDIES ON SERRATIA MARCESCENS L-ASPARAGINASE

A.A. Khan, S.P. Pal, S.R.V. Raghavan and P.K. Bhattacharyya.

Central Research Laboratory, Antibiotics Plant, Indian Drugs and Pharmaceuticals Ltd., Virbhadra (Rishikesh), India.

Received September 14, 1970

Summary. Cells of S. marcescens showed induced formation of L-asparaginase by its substrate, L-asparagine. The cells also showed nutritional requirement of glutamic acid, methionine and glucose for optimal production of enzyme. The enzyme was partially purified from both induced and non-induced cells. The differences in kinetic parameters and electrophoretic mobilities demonstrated the presence of two isoenzymes in non-induced preparations. The induced preparations showed only one enzyme, distinct from the two non-induced isoenzymes.

Isoenzymes of L-asparaginase (L-asparagine amido hydrolase, E.C. 3.5.1.1) are known in the case of E. coli (1-4) and M. tuberculosis (5). However, no such data are available for the Serratia marcescens enzyme. To our knowledge this is a first report of substrate induction for L-asparaginase.

Methods. The strain of Serratia marcescens Nima was maintained at 27°C for 24 hr on slops of nutrient agar. The organisms were grown in aerobic conditions in 500 ml shake flasks at 27°C for 22 hr. For induction L-asparagine was added 2 hr after inoculation, with cells of S. marcescens grown in a synthetic medium.

Corn steep medium was prepared as described by Roberts et al. (6). A nutrient broth containing 1% peptone and 0.5% each of beef and yeast extracts and NaCl was employed for growing non-induced cells. The basal synthetic medium consists of : 3.0 g KH_2PO_4, 7.0 g Na_2HPO_4, 1.2 g $MgSO_4$, 1.0 g NaCl, 1.0 mg $FeCl_3$ $6H_2O$ and 1 g glucose in a liter of distilled water. The medium was supplemented with L-asparagine (final concentration, 0.3%). The

schedule of addition of L-glutamic acid and phenylacetamide in induction studies was similar to that described for L-asparagine.

The cells were centrifuged in cold, washed with normal saline and uniformly suspended in 0.05M phosphate buffer, pH-7.5.

The enzyme activity was assayed according to the procedure of Meister (7); the reaction was run at 37°C for 30 min and the liberated ammonia was estimated by Nesslerization. A unit of enzyme caused a liberation of 1 u mole of ammonia under the assay conditions. Protein was estimated by the method of Lowery et al. (8).

For the purification of L-asparaginase S. marcescens cells were sonified for 12 min in a Branson sonifier, acetonised and the powder dried under vacuum. The enzyme was extracted by suspending the acetone powder for 1 hr in 0.1 M phosphate buffer, pH-7.5. The supernatant obtained after centrifugation was subjected to ethanol fractionation. To each volume 0.5 volume of chilled ethanol was added, centrifuged after 1 hr and the precipitate discarded. To the supernatant chilled ethanol was again added to make upto 1.25 volume of ethanol. The resulting suspension was centrifuged after 1 hr and the precipitate was suspended in a minimal volume of 0.02 M phosphate buffer, pH-7.5 and recentrifuged. The supernatant containing all the L-asparaginase activity was applied to a DEAE-cellulose column (18x1.5 cm) pre-equilibrated with 0.02 M phosphate buffer, pH-7.5. The adsorbed enzyme was fractionated by gradient elution employing 0.02 M phosphate buffer, pH-7.5 and 0.5 M NaCl. The active eluate fractions were subjected to negative gel treatment on calcium phosphate gel at a protein gel ratio of 1:1.

The electrophoresis was carried out for 18 hr with 0.075M citrate phosphate buffer, pH-7.0, at 8 V/cm in starch gel prepared in 0.013M of the same buffer. The gel was then uniformly

segmented and after elution assayed for localization of enzyme activity.

Results. Induction studies : Cells of S. marcescens grown in the synthetic medium produced at least 3 - 8 times more L-asparaginase with L-asparagine as a inducer. The optimum concentration of the inducer was found to be 0.3%. It was observed that a single initial dose of the inducer produced only 70% of the enzyme activity attainable with cells receiving the same amount of inducer in three equal doses. Comparative studies on induced cells of S. marcescens grown in the presence of L-asparagine, L-glutamic acid and phenylacetamide (each at 0.3% concentration) revealed that the production of enzyme was best maintained both by L-asparagine and L-glutamic acid. Phenylacetamide, on the other hand proved to be a powerful inhibitor. Between L-asparagine and L-glutamic acid, the production response was 30% more with L-glutamic acid. Studies on the induction level of enzyme with different concentrations of L-glutamic acid established that the production of enzyme as well as cell growth was optimum at 0.3%. Subdividing the L-glutamic acid dose of 0.3% in three equal portions did not, however, result in increased production of enzyme, as in the case of L-asparagine. To evaluate, the relative inducer efficiency of L-glutamic acid and L-asparagine, the cells of non-induced organism were grown in presence of both the amino acids. L-asparagine acted as an efficient inducer, whereas L-glutamic acid failed to induce the formation of the enzyme.

In nutritional studies the supplementation of L-asparagine medium with amino acids such as L-methionine and L-glutamic acid resulted in increase production of enzyme. Supplementation of sugars such as glucose, maltose, sucrose and lactose enhanced the production of enzyme and glucose gave the best response. Cells

Table 1. Purification of non-induced and induced L-asparaginases from cells of S. marcescens.

Step	Total activity units	Total protein mg	Specific activity units/mg protein	Total activity units	Total protein mg	Specific activity units/mg protein
	Non-induced			Induced		
Sonicated cell suspension	2,400.0	2,609	0.9	5,040.0	1,506	3.4
Acetone powder extract	2,028.4	392	5.2	4,228.0	350	12.8
Ethanol extract	1,624.0	140	11.6	3,225.0	150	21.5
DEAE-cellulose fraction	887.6	11.4	78.4	1,657.0	14	118.0
Calcium phosphate gel	710.0	6.8	104.4	1,326.4	8.4	158.0

grown in corn steep medium produced at least twice as much enzyme as those grown in the synthetic medium. Supplementation of the corn steep medium with 1% molasses or 0.1% glucose caused a further increase of 20% in enzyme activity.

Partial purification : The profiles of the purification procedure from both non-induced and induced organisms are described in Table 1. With the procedure adopted a 113 and 47 - fold purified preparations were obtained from non-induced and induced organisms respectively. Enzyme elution from DEAE-cellulose gave a single peak both in non-induced and induced preparations. Negative gel treatment on calcium phosphate enriched the enzyme to a considerable extent. Positive adsorption on the gel was unsuitable because it resulted in complete inactivation of induced as well as non-induced enzymes.

Electrophoretic studies of the non-induced and induced enzyme preparations clearly revealed the presence of three distinctly different proteins with enzyme activity.

Kinetic studies : The rate of the reaction was linear upto 80 min with non-induced and 60 min with induced preparations.

The pH-rate profile of the preparation from non-induced organism showed two activity peaks; one at pH-7.0 and the other at the pH-8.5. However, the preparations from the induced organism showed only one peak at pH-8.0.

Usual Michaelis type of substrate kinetics was observed both with the non-induced and the induced enzymes. Double reciprocal plots gave Km values of 2×10^{-3} M and 8.3×10^{-4} M respectively for pH-7.0 and 8.5 non-induced enzymes and 2.1×10^{-4} M for induced enzyme.

L-cysteine inhibited the L-asparaginase activities of both the preparations. A differential type of cysteine inhibition

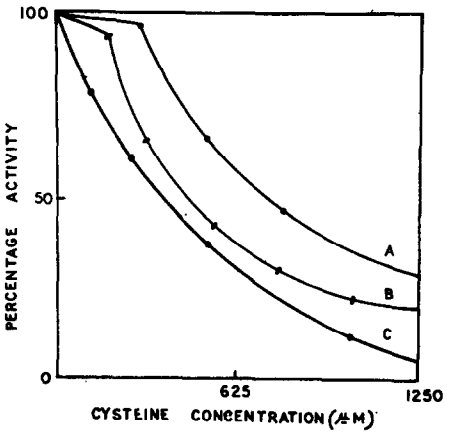

Fig. 1. Inhibition of L-asparaginases by cysteine
A-induced enzyme; B-pH-7 non-induced enzyme;
C-pH- 8.5 non-induced enzyme.

was observed in pH-7.0 and 8.5 non-induced enzymes as well in the case of the induced enzyme. Plots of cysteine concentration and percent activities (Fig. 1) gave sigmoidal responses with the pH-7.0 non-induced enzyme and the pH -8.0 induced enzyme. The pH-8.5 non-induced enzyme showed a regular hyperbolic curve. The sigmoidal shape of the curves suggest allosteric inhibition by cysteine. A plot of the cysteine inhibition of the pH-7.0 non-induced enzyme, and the induced enzyme in Hill equation (9-11) gave straight lines (Fig. 2) with values of interaction coefficients (n) equal to 1.6 for the induced and 1.4 for the pH-7.0 non-induced enzyme. These values of interaction coefficients may be explained by assuming atleast two binding sites, if not more, for cysteine. The K values for the induced and the pH-7.0 non-induced enzymes were 5.2 m M and 4.0 m M respectively.

Other amino acids such as L-aspartic acid, L-glutamic acid, L-methionine and L-valine did not effect the activities of none of the preparations.

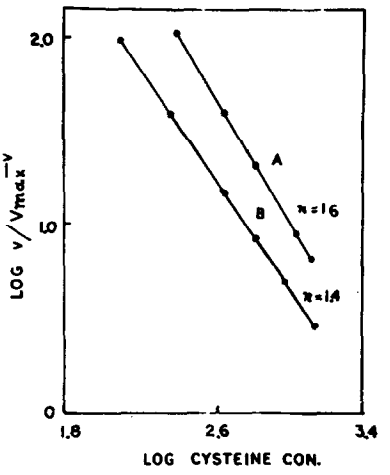

Fig. 2. Hill plot of cysteine inhibition.
A- induced enzyme; B- pH-7 non-induced enzyme.

Preparations of L-asparaginase from induced organism were quite stable in cold or frozen conditions, however, non-induced preparations were comparatively unstable. Even the addition of L-asparagine did not impart stability to these preparations.

Discussion. Induction of L-asparaginase by its substrate, L-asparagine, in S. marcescens cells as well as the specificity of inducer for induced production of enzyme is an indication of a regulator type control of L-asparaginase. L-glutamic acid which does not induce enzyme production in non-induced cells, however, sustains the production of the induced L-asparaginase. The mechanism of this maintenance is not clear.

Nutritional studies showed increase enzyme production in the presence of glucose which are in contrast to E. coli enzyme (6,12) where the addition of glucose caused decrease in enzyme production.

The differences in pH, Km, cysteine inhibition, stability and electrophoretic mobility suggest that these three molecular species of L-asparaginase are distinctly different iso-enzymes.

Cysteine, a negative allosteric effector (13,14) of the induced enzyme and the pH-7.0 non-induced enzyme, appears to regulate these activities by modifying the affinity for L-asparagine at the catalytic site. The inhibition of all the three isoenzymes by cysteine could well be a feed back type as proposed in M. tuberculosis (5) but in contrast the enzymes of the S. marcescens were uneffected by L-aspartic acid.

Acknowledgements. The authors are thankful to Dr. P.P. Mukherji for his suggestions and help during the work and to the skillfull technical assistance of Mr. A.K. Goel and Mr. S.K. Mehandiratta.

REFERENCES

1. Schwartz, J.H., Reeves, J.Y. and Broome, J.D., Proc. Natl. Acad. Sci. U.S., 56, 1516 (1966).

2. Roberts, J., Prager, M.D. and Bachynsky, N., Cancer Res., 26, 2213 (1966).

3. Champbell, H.A., Mashburn, L.T., Boyse, E.A., and Old, L.J., Biochemistry, 6 , 721 (1967).

4. Cedar, H., and Schwartz, J.H., J. Biol. Chem., 242, 3753 (1967).

5. Jayaram, H.N., Ramakrishnan, T., and Vaidynnathan, C.S., Arch. Biochem. Biophys., 126, 165 (1968).

6. Roberts, J., Burson, G., and Hill, J.M., J. Bacteriol., 95, 2117 (1968).

7. Meister, A., Methods Enzymol., 2, 383 (1955).

8. Lowry, O.G., Rosenbrough, N.J., Farr, A.L., and Randall R.J., J. Biol. Chem., 193, 265 (1951).

9. Changeux, J.P., Cold Spring Harbor Symp. Quant. Biol., 28, 497 (1963).

10. Atkinson, E.D., Hathaway, J.A. and Smith, E.C., J. Biol. Chem., 240, 2682 (1965).

11. Taketa, K., and Pogell, B.M., J. Biol. Chem., 240, 651 (1965).

12. Cedar, H., and Schwartz, J.H., J. Bacteriol., 96,2043 (1968).

13. Changeux, J.P., and Jacob, F., J. Mol. Biol., 6, 306 (1963).

14. Monod, J., Wyman, J., and Changeux, J.P., J. Mol. Biol., 12, 88 (1965).