BBA 66771

PURIFICATION AND PHYSICOCHEMICAL PROPERTIES OF L-GLUTAMINASE FROM *PSEUDOMONAS*

HIDEO KATSUMATA[a], RYOICHI KATSUMATA[a], TSUTOMU ABE*, OSAMU TAKENAKA* AND YUJI INADA*

**Laboratory of Biological Chemistry, Tokyo Institute of Technology, Ookayama, Meguroku, Tokyo, and [a]Fuji Research Laboratory, Kyowa Hakko Kogyo Co. Ltd., Nagaizumi-cho, Shizuoka-ken (Japan)*

(Received June 16th, 1972)

SUMMARY

Crystalline L-glutaminase (L-glutamine amidohydrolase, EC 3.5.1.2) was obtained from *Pseudomonas* and its homogeneity was confirmed by electrophoresis and ultracentrifugal analysis. The specific activity of the enzyme was 36 I.U. per mg of protein for L-glutaminase action and 15 I.U. for L-asparaginase action at pH 7.0. Physicochemical properties and amino acid composition of the enzyme were established.

INTRODUCTION

Considerable attention on the physiological function of glutaminase (L-glutamine amidohydrolase, EC 3.5.1.2) and asparaginase(L-asparagine amidohydrolase, EC 3.5.1.1) in terms of remission in human leukemia[1,2], has resulted in extensive studies on their physicochemical properties[3–6] and amino acid residues in asparaginase related to their enzymic activities[7,8]. Recently, glutaminase from *Achromobacteraceae* with antitumor activity was isolated in a crystalline form[5]. Soda *et al.*[6] obtained a crystalline glutaminase from *Pseudomonas aeruginosa* and found two isozymes having molecular weights of 137 000 and 67 000. The present study deals with isolation, crystallization and physicochemical properties of the enzyme.

EXPERIMENTAL

Pseudomonas p-210 (ATCC 21025) was used as a source of L-glutaminase. The cells were grown in a medium containing 1% casamino acid, 0.5% L-glutamine, 0.1% KH_2PO_4, 0.1% Na_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, minerals an vitamins at pH 7.2. The cultivation was carried out at 30 °C for 12–16 h under aeration. Protein concentration was determined by the method of Lowry *et al.*[9]. L-Glutaminase activity was deter-

* Postal address.

mined by anion-exchange chromatographic method[10], or by the hydroxamate method[11]. L-Asparaginase activity was determined by direct nesslerization of NH_3 produced by the enzymic action. One international unit (I.U.) of the activity is defined as that amount of enzyme which catalizes the formation of 1 μmole of ammonia per min at 37 °C. Ultracentrifugation experiments were performed with a Beckman model E ultracentrifugal analyzer. Thin-layer filtration was performed by the method of Radola[12]. Amino acid determination was carried out for 16, 24, 36, 48 or 72 h hydrolysates (at 110 °C with 5.7 M HCl) with a Hitachi amino acid analyzer model KLA-3.

RESULTS AND DISCUSSION

Pseudomonas cells were ruptured with a Manton Gaulin homogenizer at pH 6.0 and then centrifuged. To the supernatant, 2 vol. of chilled acetone were slowly added and the resultant precipitate was dissolved in 10 mM K_2HPO_4 with 50 mM aspartate. The pH of the clear solution was adjusted to 9.0 with alkali, and 3% acrinol was added up to 0.014% (w/v). The solution was kept at 50 °C for 30 min, cooled rapidly and centrifuged in order to remove precipitates. Acrinol was completely removed by charcoal treatment. Ammonium sulfate was added up to 60% saturation to the clear supernatant. The precipitate formed was dissolved in a small volume of 10 mM Tris–HCl buffer (pH 8.0) with 50 mM aspartate and dialyzed against the same buffer solution. The dialysate was applied on DEAE cellulose column equilibrated with 10 mM Tris–HCl buffer (pH 8.0) with 10 mM aspartate. The active fractions, which possess the glutaminase activity, were collected, and 2 vol. of chilled acetone were slowly added. The resultant precipitate was dissolved in a small volume of Tris–HCl buffer (pH 7.0). Ammonium sulfate was added up to 15% saturation and the precipitate was removed by centrifugation. To the supernatant, ammonium sulfate was gradually added up to 60% saturation of the salt and a faint turbidity appeared in the solution. On standing overnight at 5 °C, crystalline glutaminase was formed. The crystals were rhombic plates, as shown in Fig. 1. Table I summarizes the procedure of the crystallization of glutaminase and the specific activity at each step. The specific activity was determined by the hydroxamate method and the values obtained

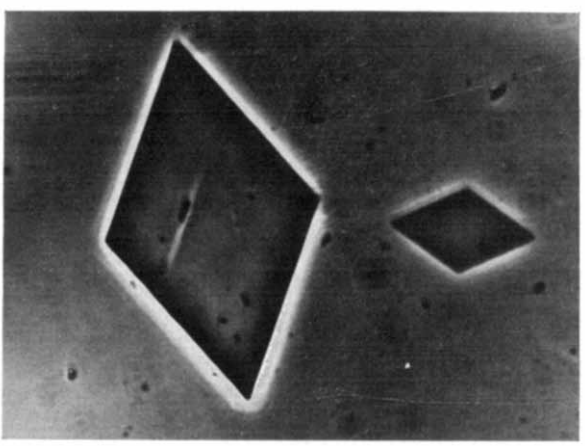

Fig. 1. Crystalline L-glutaminase from *Pseudomonas* p-210(ATCC 21025). × 300.

TABLE I

PURIFICATION PROCEDURE OF L-GLUTAMINASE FROM *Pseudomonas* p-210 (ATCC 21025)

	Protein (g)	*Glutaminase activity (I.U.)*	*Specific activity (I.U./mg)*	*Yield (%)*
Fermentation broth	4900	$17.6 \cdot 10^5$	0.36	100
Homogenate	1320	11.4	0.86	65
Acrinol	182	5.3	2.9	30
DEAE cellulose	12.1	3.3	27	19
Crystallization	7.0	2.7	38	15
Recrystallization	5.9	2.1	36	12

were converted to I.U. The rate of the hydroxamate formation is about 1.7 times faster than that of the hydrolysis of the amide of L-glutamine at pH 8.0.

The ultracentrifugation pattern of the enzyme shows a single symmetrical peak (Fig. 2). Electrophoresis with cellulose acetate shows only one sharp and strong band. The isoelectric point of the enzyme preparation was determined to be pH 7.6 by the isoelectric focusing method, using carrier ampholite (pH 7–10). The absorbance at 280.5 nm of a 1% enzyme solution was 8.65. L-Glutaminase activity was found to be 36 I.U. per mg of protein at pH 6.0–8.0 (optimum pH), and L-asparaginase activity was 15 I.U. at pH 7.0 and 28 I.U. at pH 10.5 (optimum pH). This enzyme is classfied in the second group of glutaminases proposed by Hartman[13], because it has hydroxamate activity and acts upon D- as well as L-glutamine and upon asparagine.

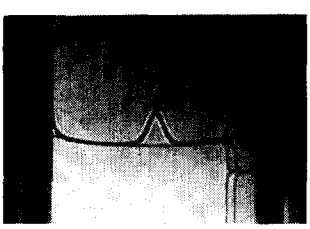

Fig. 2. Ultracentrifugation pattern of L-glutaminase. Photograph was taken 36 min after attaining a rate of 60 000 rev./min. Glutaminase concentration was 0.7% in 0.01 M phosphate buffer (pH 6.8) containing 0.5 M NaCl.

The molecular weight of glutaminase was determined to be 122 000 ± 10 000 by thin-layer Sephadex G-200 (super fine) chromatography (phosphate buffer, pH 7.0, $I = 0.1$), using chymotrypsinogen, serum albumin, asparaginase, γ-globulin, fibrinogen and thyroglobulin as standards. Equilibrium ultracentrifugation was carried out at a concentration of 0.1 or 1.0 mg protein per ml. Equilibrium was attained well within 35 h at a rotor speed of 17 820 rev./min (phosphate buffer, pH 7.0, $I =$ 0.1, at 20 °C). The molecular weight was calculated according to the method of Yphantis[14] and found to be 118 000. The partial specific volume, 0.732, was calculated from the amino acid composition of the enzyme[15].

The amino acid contents were determined by the method of Moore and Stein[16]. The second column in Table II shows the contents of each amino acid in weight per-

cent obtained from the data at different hydrolysis periods. The third and fourth columns show the comparison of molar contents of amino acids of glutaminase from *Pseudomonas* with those for *Achromobacteraceae* reported by Roberts *et al.*[5]. The contents of aromatic and basic amino acids in *Pseudomonas* enzyme were considerably lower than those in the *Achromobacteraceae* enzyme, while the content of glutamic acid was higher than that in the *Achromobacteraceae* enzyme.

TABLE II

AMINO ACID CONTENTS IN L-GLUTAMINASE FROM *Pseudomonas* AS COMPARED WITH THOSE IN GLUTAMINASE FROM *Achromobacteraceae*

Contents of Ser, Thr, Asp and Lys were obtained by extrapolation of zero hydrolysis time, contents of Val, Leu and Ile by extrapolation to 72 h of hydrolysis and other amino acids as a mean value of the data obtained at various hydrolysis times.

Amino acid	*% wt*	*Number of residues per 1000*	
		From Pseudomonas	*From Achromobacteraceae*
Lys	8.53	71.3	77
His	1.92	15.0	29
Arg	5.55	38.1	33
Asp	13.15	122.5	121
Thr	5.07	53.8	53
Ser	5.13	63.1	62
Glu	11.50	95.5	77
Pro	2.57	28.4	34
Gly	4.41	82.9	73
Ala	7.42	112.0	119
Val	9.85	106.3	94
Met	3.05	24.9	24
Ile	5.60	53.0	54
Leu	8.68	82.2	79
Tyr	2.99	19.7	26
Phe	2.66	19.4	30
Cys*	0	0	0
Trp**	1.95	11.2	14
	100.03	999.3	998

* Measured as cysteic acid after performic oxidation[17].
** Measured spectrophotometrically[18].

ACKNOWLEDGMENT

The authors are grateful to Dr K. Soda of Kyoto University for his valuable suggestions for obtaining crystalline glutaminase and Dr T. Nagumo and Dr S. Ishii of Kitasato University for carrying out the ultracentrifugal analysis.

REFERENCES

1 E. N. Hersh, *Science*, 172 (1971) 736.
2 E. Grundmann and H. F. Oettgen, *Experimental and Clinical Effects of* L-*asparaginase*, Springer-Verlag, 1970.
3 A. Arens, E. Rauenbusch, E. Irion, O. Wagner, K. Bauer and W. Kaufmann, *Hoppe-Seyler's Z. Physiol. Chem.*, 351 (1970) 197.

4 N. Nakamura, Y. Morikawa, T. Fujio and M. Tanaka, *Agric. Biol. Chem.*, 35 (1971) 219.
5 J. Roberts, J. S. Holcenberg and W. C. Dolowy, *J. Biol. Chem.*, 247 (1972) 84.
6 K. Soda, M. Ohshima and T. Yamamoto, *Biochem. Biophys. Res. Commun.*, 46 (1972) 1278.
7 Y. Nishimura, H. Makino, O. Takenaka and Y. Inada, *Biochim. Biophys. Acta*, 227 (1971) 171.
8 H. Makino, O. Takenaka and Y. Inada, *Biochim. Biophys. Acta*, 263 (1972) 477.
9 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
10 T. Abe, Y. Tamaura, Y. Nishimura, O. Takenaka and Y. Inada, *J. Chromatogr.*, 69 (1972) 388.
11 F. Lipmann and L. C. Tuttle, *J. Biol. Chem.*, 159 (1945) 21.
12 B. J. Radola, *J. Chromatogr.*, 38 (1968) 61.
13 S. C. Hartman, *Enzymes.*, 4 (1971) 79.
14 D. A. Yphantis, *Biochemistry*, 3 (1964) 297.
15 T. L. McMeekin, M. L. Groves and N. J. Hipp, *J. Am. Chem. Soc.*, 71 (1949) 3298.
16 S. Moore and W. H. Stein, *Methods Enzymol.*, 6 (1963) 819.
17 C. H. W. Hirs, S. Moore and W. H. Stein, *J. Biol. Chem.*, 219 (1956) 611.
18 H. Edelhoch, *Biochemistry*, 6 (1967) 1948.