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REINVESTIGATION OF THE PHYSICOCHEMICAL AND ENZYMIC PROPERTIES OF L-GLUTAMINASE FROM *PSEUDOMONAS*

TSUTOMU ABE, OSAMU TAKENAKA and YUJI INADA

Laboratory of Biological Chemistry, Tokyo Institute of Technology, O-okayama, Meguro-ku, Tokyo (Japan)

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SUMMARY

In a previous study ((1972) Biochim. Biophys. Acta 289, 405–409) L-glutaminase (EC 3.5.1.2) was crystallized from *Pseudomonas* (ATCC 21025). The specific activity of this enzyme decreased rather rapidly during incubation in aqueous solution. In the present study, isoelectric focusing, together with disc electrophoresis, revealed that the incubation resulted in the cleavage of the enzyme probably due to contamination by traces of protease(s). The physicochemical and enzymic properties were reinvestigated. The specific activity of the enzyme at pH 7.0 was 84 I.U. per mg of protein for L-glutaminase activity and 73 I.U. for L-asparaginase activity.

INTRODUCTION

In a previous study [1], L-glutaminase (EC 3.5.1.2) was crystallized from *Pseudomonas* (ATCC 21025) and its homogeneity was confirmed by electrophoresis and ultracentrifugal analysis. The specific activity of this enzyme decreased rather rapidly when incubated in aqueous solution at 37 °C. In the present study, the inactivation was examined by isoelectric focusing and disc electrophoresis, and the physicochemical and enzymic properties of L-glutaminase were reinvestigated.

MATERIALS AND METHODS

L-Glutaminase was isolated from freshly harvested cells of *Pseudomonas* species (ATCC 21025) and was crystallized two times as described previously [1]. The crystals were stored at 4 °C as a suspension in $(NH_4)_2SO_4$ solution of 60% saturation. γ-Glutamyl hydroxamate and carrier ampholyte (pH 7–9) were purchased from Sigma Chemical Co. and LKB Instruments, respectively.

Isoelectric focusing was performed at 500 V for 4 days. Disc gel electrophoresis in the presence of sodium dodecylsulfate was carried out by the method of Weber and Osborn [2], using bovine serum albumin, egg albumin, *Escherichia coli* asparaginase, bovine chymotrypsinogen and horse heart cytochrome *c* as standards. The R_F values of protein bands were measured with a Gilford 2400-S linear transport spectrophotometer. Thin-layer gel filtration, using Sephadex G-200 (superfine)

equilibrated with 0.1 M phosphate buffer (pH 7.0), was performed by the method of Radola [3]. Protein standards used were bovine γ-globulin, asparaginase, bovine serum albumin and egg albumin.

Protein concentration was determined by the method of Lowry et al. [4], using bovine serum albumin as a standard. L-Glutaminase and L-asparaginase activities of this enzyme were determined by direct nesslerization according to the method of Roberts et al. [5], by measuring the absorbance at 425 nm. The hydroxamate method was used for the routine assay of the enzymic activity. The assay mixture contained 20 mM glutamine and 0.3 M neutralized hydroxylamine in 20 mM phosphate buffer (pH 7.0) and enzyme of an appropriate concentration. γ-Glutamyl hydroxamate was determined by the method of Lipmann and Tuttle [6].

RESULTS AND DISCUSSION

Fig. 1 shows the result of isoelectric focusing of L-glutaminase. Curve A shows that L-glutaminase was separated into three components. These are designated as Component I, II and III, respectively, Component I having the highest pI value and

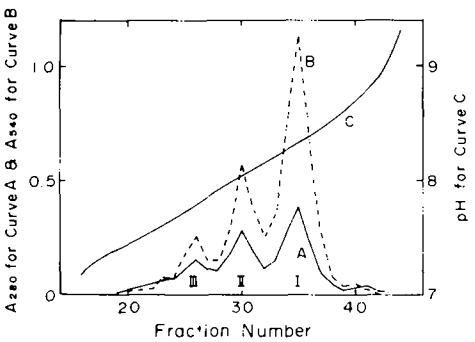

Fig. 1. Isoelectric focusing electrophoresis of L-glutaminase. The absorbance value at 280 nm (Curve A) measured for protein concentration, and at 540 nm (Curve B) for hydroxamate activity and the pH value (Curve C) of each fraction plotted against fraction number.

Component III the lowest. The specific activity of Component I using L-glutamine as the substrate, was 84 I.U. per mg, which was much higher than that reported previously (36 I.U. per mg). The specific activity decreased progressively from Component I to Component III. The values of isoelectric points, L-glutaminase and L-asparaginase activities and hydroxamate activity of each component are listed in Table I.

TABLE I

Isoelectric point of Components I, II and III, and the specific activities of these components at pH 7.0, using L-glutamine (Glutaminase) and L-asparagine (Asparaginase) as substrates and using the glutamyl hydroxamate (Hydroxamate) method. Activities are expressed as I.U. per mg of protein.

Component	I	II	III
Isoelectric point	8.35	8.05	7.80
Glutaminase	84	68.5	60.5
Asparaginase	73	58.5	54
Hydroxamate	105	74	58.5

A single fraction of each component was analyzed by polyacrylamide disc gel electrophoresis in the presence of sodium dodecylsulfate and the results are shown in Fig. 2. The gel of Component I shows one intense sharp band and its apparent molecular weight was 36 400 ± 1000. The disc gel electrophoretic pattern of L-glutaminase, prepared by dissolving the crystals directly into sodium dodecylsulfate solution, showed one intense sharp band and its R_F value was identical with that of Component I. Therefore, Components II and III are assumed to be generated from Component I during electrophoresis. Thin-layer gel filtration of Component I revealed that the apparent molecular weight of the component was 130 000 ± 10 000.

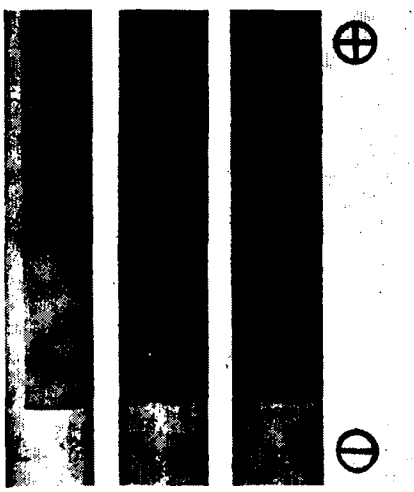

Fig. 2. Polyacrylamide gel disc electrophoretic patterns. Gels I, II and III are obtained for Components I, II and III of Fig. 1, respectively.

L-Glutaminase may be composed of four identical subunits of molecular weight 36 400 and its apparent molecular weight may be calculated to be 146 000. This value is higher than that obtained in the previous study, where the molecular weight of L-glutaminase was determined to be 122 000 ± 10 000 by thin-layer gel filtration and 118 000 by equilibrium centrifugation. The gels of Components II and III show two protein bands. The upper band in each gel corresponds to the band of Gel I (36 400); the lower bands of Gels II and III have the same R_F value and their apparent molecular weights were calculated to be 32 700.

L-Glutaminase solution was incubated at 37 °C for 2 h at pH 7.0 and was subjected to isoelectric focusing electrophoresis. The incubated glutaminase was also separated into three components, and the pI values of these components are identical with the corresponding component shown in Fig. 1. However, the relative concentration of Component I decreased and those of Components II and III increased. This result confirms that the incubation induced the conversion of the subunit of molecular weight 36 500 of Component I into the subunit of molecular weight 32 700.

The effects of the incubation of L-glutaminase solution on the enzymic activity were examined under various conditions and the results are shown in Table II. The incubation of L-glutaminase solution at pH 7.0 and at 37 °C induced the loss of

TABLE II

Effects of incubation for 15 h at pH 7.0 of L-glutaminase solution on its enzymic activity.

Reagents added	Concn (mM)	Residual activity (%) after incubation	
		37 °C	4 °C
None		7	100
EDTA	1	102	106
EGTA	1	80	100
Ca^{2+}	1	0	99
Mg^{2+}	1	9	95

activity to 7% of its original activity. The presence of a chelating agent such as EDTA or disodium ethyleneglycol-bis-(β-aminoethyl ether) *N,N'*-tetraacetic acid (EGTA) reduced the inactivation. Full activity was also retained when the temperature was kept at 4 °C during incubation. The presence of Ca^{2+} or Mg^{2+} slightly enhanced the inactivation. These results may indicate the contamination by traces of protease(s) for which metal ion(s) is essential. Indeed, no loss of activity of Component I was observed upon incubation at 37 °C for 1 day. Isoelectric focusing electrophoresis may have removed the protease(s).

The present results suggest the contamination of L-glutaminase by traces of protease(s) which could not be detected by the usual analytical methods for proteins. The protease(s) may cleave L-glutaminase at a particular peptide bond(s), which may be labile to proteolytic digestion since intermediates, Components II and III, were detected by isoelectric focusing electrophoresis. Some of the previous experiments [1], therefore, may have been done on the intermediates. The presence of the intermediates will force us to examine the possibility that the isozymes which we found were, in fact, derived from a single enzyme due to the presence of traces of protease during isolation.

The crystallization of L-glutaminase from *Pseudomonas* [1, 7] and *Acinetobacter* [5] will lead us to the application of this enzyme as an antineoplasmic agent [8]. Attention should be paid to the presence of a labile peptide bond(s) in this enzyme when the blood clearance of the enzyme is high.

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