LIMITED PROTEOLYSIS OF *PSEUDOMONAS* GLUTAMINASE BY PORCINE TRYPSIN

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1. Introduction

Glutaminase (glutamine amidohydrolase, EC 3.5.1.2) was crystallized from *Pseudomonas* (ATCC 21025) and its enzymic and physicochemical properties were investigated [1,2]. This enzyme is comprised of four identical subunits of mol. wt 36 400 and isoelectric point was 8.35. Spec. act. at pH 7.0 was 84 IU per mg of protein for L-glutaminase activity and 73 IU for L-asparaginase activity.

The previous study [2] suggested protease(s) contaminating glutaminase crystals cleaved the enzyme at a particular peptide bond(s). The limited proteolysis resulted in the decrease of enzymic activity and of isoelectric point. The present paper deals with limited proteolysis of glutaminase by a well-known protease, porcine trypsin.

2. Materials and methods

Glutaminase was crystallized as described previously [2] and its enzymic activity was assayed by the hydroxamate method. The previous study suggested that the mother liquor of the crystalline glutaminase was contaminated by traces of protease(s) which could not be detected by the usual analytical methods for proteins. Glutaminase crystals were extremely separated from the mother liquor in each crystallization and the number of crystallizations was raised from two times to three times. These procedures may exclude the protease(s). No loss of enzymic activity of glutaminase was observed upon incubation of its aqueous solution. Porcine pancreatic trypsin and soybean trypsin-inhibitor were purchased from Novo Industry and Miles, respectively.

Disc gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to the method of Weber and Osborn [3], using bovine serum albumin, egg albumin, chymotrypsinogen and egg white lysozyme as standards. Gel filtration of trypsin-digested glutaminase was carried out on a Sephadex G-100 column (2.5 × 40 cm) equilibriated with 10 mM collidine-acetate buffer (pH 7.0). Protein and peptide components of the digested glutaminase were separated from each other by gel filtration using a Sephadex G-200 column (2.5 × 40 cm) equilibriated with 1% sodium dodecyl sulfate.

N-Terminal amino acid was determined according to the procedure of Gray [4] and Woods and Wang [5] using 1-dimethylaminonaphthalene-5-sulfonyl chloride. C-Terminal amino acid was determined by the digestion of glutaminase (15 mg/ml) with carboxypeptidase A (0.3 mg/ml) at 30°C in 0.1 M phosphate buffer (pH 7.5) in the presence of 2 M urea.

Tryptic digestion of glutaminase (5 mg/ml) was carried out with porcine trypsin (0.1 mg/ml) at 30°C in 50 mM phosphate buffer of pH 7.0 and was terminated at desired time by 2 mol of trypsin inhibitor per mole of trypsin. For preparation of the digested glutaminase on a large scale, the concentration of glutaminase was 20 mg/ml and the ratio of trypsin to glutaminase was 1:100.

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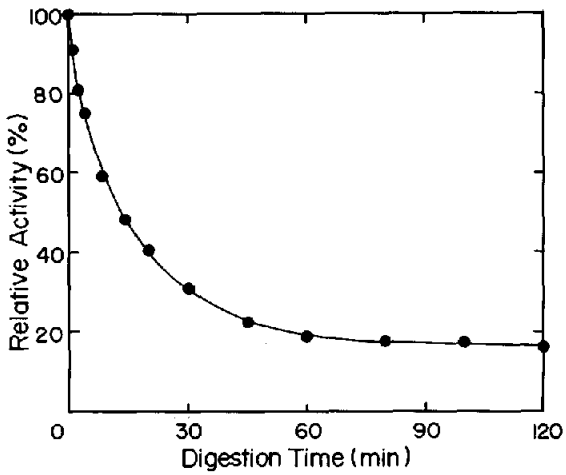

Fig.1. Tryptic digestion of glutaminase. Residual enzymic activity plotted against incubation time.

3. Results

Enzymic activity of glutaminase decreased upon incubation with trypsin and reached a constant level of 16% of its original activity (fig.1). Patterns of disc gel electrophoresis of glutaminase in the presence of sodium dodecyl sulfate changed parallel to the loss of its enzymic activity (fig.2). Trypsin cleaved the glutaminase molecule into protein and peptide components. The protein component was named T-protein and the peptide component T-peptide. The mol. wt of the native subunit of glutaminase was assumed to be 36 400 and that of T-protein to be 31 700. The area of each protein band was determined from the densitometric traces of these gels. The ratio of the area of native subunit of glutaminase at zero time incubation to that of T-protein after 2-hr incubation was 100:95.

The trypsin-digested enzyme has the same mobility as that of native enzyme on a thin layer gel chromatogram using Sephadex G-200 super fine [6], and any ninhydrin positive spots having low mobility could not be detected. Moreover, the digested enzyme was eluted as a single component at the void volume of a Sephadex G-100 column without any components of low mol. wt. These results indicate that the digested glutaminase is still comprised of four subunits and that T-peptide is not released from the digested enzyme.

The trypsin-digested enzyme was separated into 2 components by gel chromatography in the presence of 1% sodium dodecyl sulfate (fig.3). The first component was identified as T-protein by disc gel electrophoresis in the presence of sodium dodecyl sulfate. The second component was labelled with fluorescamine and was applied to disc gel electrophoresis in the presence

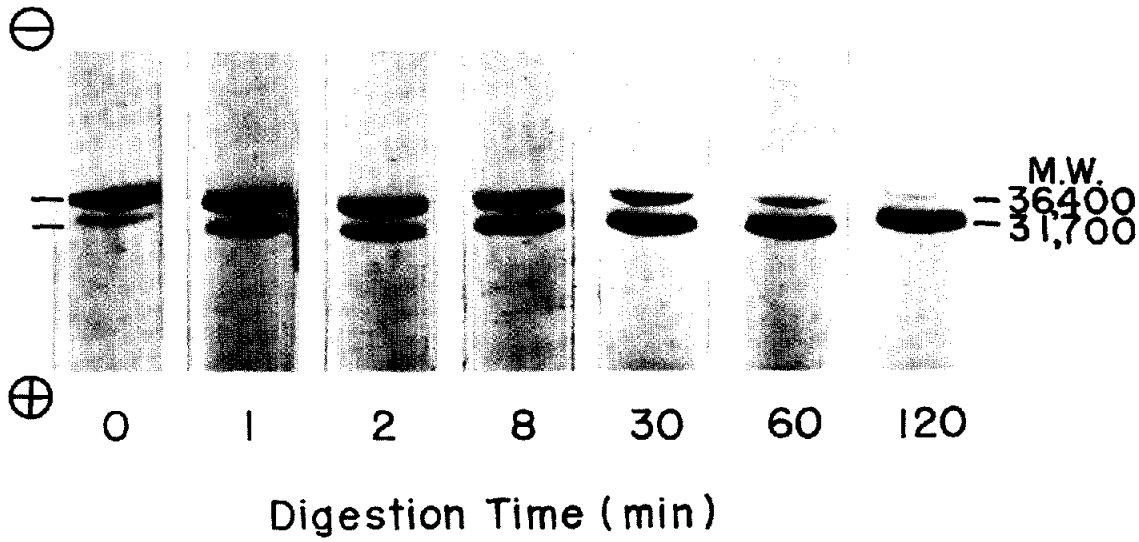

Fig.2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis patterns of trypsin-digested glutaminase.

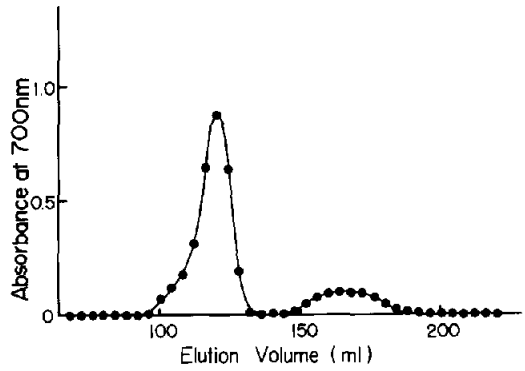

Fig.3. Gel filtration of trypsin-digested glutaminase using Sephadex G-100 super fine equilibriated with 1% sodium dodecyl sulfate. Column: 2.5 × 40 cm.

of sodium dodecyl sulfate. Only one rapidly moving band was detected under 365 nm light. The second component was, therefore, considered to be T-peptide. The protein in the shoulder at the frontal side of the first component was the native subunit of glutaminase.

N-Terminal amino acids of native subunit, T-protein and T-peptide were determined using 1-dimethylamino-naphthalene-5-sulfonyl chloride and found to be lysine for both of native glutaminase and T-peptide, and leucine for T-protein. T-Peptide, therefore, may be derived from the amino terminal region of glutaminase.

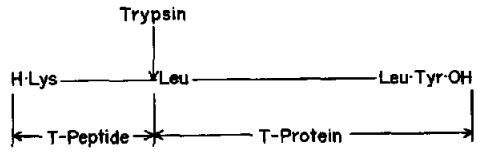

Fig.4. Location of T-protein and T-peptide in the glutaminase molecule.

Amino acids released from native glutaminase treated with carboxypeptidase are listed in table 1. Amino acid sequence at carboxy terminal region of this enzyme was assumed to be Leu-TyrOH, and these results are summarized in fig.4. Amino acid composition of native glutaminase, T-protein and T-peptide is listed in table 2.

Table 2
Amino acid contents in native subunit of glutaminase, T-protein and T-peptide

Amino acid	Number of residues per mole Native subunit	T-Protein	T-Peptide
Lys	26.9	24.7	3.7
His	5.3	5.1	0.4
Arg	13.4	13.1	1.2
Asp	40.4	36.9	5.1
Thr	18.6	13.9	1.8
Ser	23.2	19.0	1.8
Glu	33.9	29.2	6.5
Pro	9.7	8.6	0.7
Gly	27.9	22.8	4.9
Ala	37.7	26.2	8.9
Val	29.7	27.6	3.2
Met	8.3	7.9	0.5
Ile	16.1	15.2	2.4
Leu	26.2	24.2	3.2
Tyr	8.1	6.7	0.4
Phe	6.1	5.7	0.4
Cys	0[a]	0	0
Trp	4.7[b]	–[c]	0[d]
Molecular weight	36.400	31.700	4.700

[a] Determined as cysteic acid after performic acid oxidation.
[b] Determined by 3 N *p*-toluene sulfonic acid hydrolysis [7].
[c] Tryptophan content could not be determined. Amino acid content was calculated on the assumption that T-protein contains 4.7 mol of tryptophan.
[d] Determined spectrophotometrically [8].

Table 1
Amino acids released from native glutaminase upon treatment with carboxypeptidase A

Incubation time (min)	Moles of amino acid released per mole of subunit Tyr	Leu	Ala	Ile	Val
2	0.073	0.032	0.008	0.007	0
4	0.091	0.068	0.026	0.022	0
6	0.109	0.085	0.047	0.044	0
14	0.181	0.168	0.097	0.103	0.054
20	0.251	0.264	0.162	0.188	0.103
40	0.404	0.487	0.362	0.333	0.263

4. Discussion

The previous report suggested that crystalline glutaminase was contaminated by traces of protease(s) which cleaved the enzyme at a particular peptide bond(s). Parallel to the hydrolysis, mol. wt of the subunit of glutaminase was reduced from 36 400 to 32 700. Similar decrease was observed in the present study. Trypsin cleaved the glutaminase molecule at a peptide bond in the amino-terminal region and the mol. wt of the subunit of glutaminase was reduced from 36 400 to 31 700. There may exist in the glutaminase molecule a peptide chain loop which extrudes from the surface of the molecule. The contaminant protease(s) and trypsin may attack the peptide bonds on this loop.

As shown by fig.2, native subunit of glutaminase was decreased during hydrolysis by trypsin with concomitant increase of T-protein. The ratio of the area calculated from densitometric traces of native glutaminase at zero time incubation to that of T-protein after 2-hr incubation was 100:95. Color staining of glutaminase may be more intense than that of T-protein, since T-protein is a portion of the glutaminase molecule. Therefore, change of native subunit to T-protein may be in a mole to mole fashion. In other words, T-protein is not further hydrolyzed in the present condition. This fact enabled us to obtain the digested glutaminase in a homogeneous state.

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