L-Asparaginase from *Proteus vulgaris*. Subunit and Amino Acid Composition†

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ABSTRACT: Crystalline L-asparaginase from *Proteus vulgaris* has a sedimentation coefficient ($s^0_{20,w}$) of 7.9 S and a molecular weight of 120,000 (sedimentation equilibrium). The enzyme dissociates into subunits in the presence of 3 M guanidine hydrochloride, 0.1% sodium dodecyl sulfate, or 6 M urea, and loses its enzyme activity. Reconstitution and recovery of the enzyme activity are easily attained by removal of these protein denaturants. The molecular weight of the subunit is found to be 29,300 by sedimentation equilibrium in the presence of 3 M guanidine hydrochloride, 31,000–33,000 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and 30,000–35,000 by Sephadex G-150 gel filtration in the presence of 6 M urea. The minimal molecular weight is 31,400 based on amino acid composition. The NH_2-terminal amino acid is leucine and the COOH-terminal amino acid is tyrosine, respectively. Free sulfhydryl groups are not found, but one intramolecular disulfide bridge is contained in a subunit. Carbohydrate was not detected in the crystalline enzyme.

L-Asparaginase (L-asparagine amidohydrolase EC3.5.1.1) from *Escherichia coli* is used for the treatment of some human leukemias (Oettgen *et al.*, 1967; Hill *et al.*, 1969; Capizzi *et al.*, 1970). Crystallization of the enzyme from *E. coli* has been achieved in several laboratories (Ho *et al.*, 1969; Arens *et al.*, 1970; Staerk *et al.*, 1970; Nakamura *et al.*, 1971), and studies on the physicochemical properties and subunit structure have been reported (Kirschbaum *et al.*, 1969; Whelan and Wriston, 1969; Frank *et al.*, 1970; Irion and Voigt, 1970; Jackson and Handschumacher, 1970; Epp *et al.*, 1971; Glossman and Bode, 1971; Scholtan and Lie, 1971; Shifrin *et al.*, 1972).

On the other hand, we found that L-asparaginase from *Proteus vulgaris* has an antitumor activity and differs immunochemically from the *E. coli* enzyme (Tosa *et al.*, 1971). We isolated the enzyme in a crystalline form and studied some enzymic properties of the crystalline enzyme (Tosa *et al.*, 1972).

This article describes the amino acid composition and subunit structure of the crystalline L-asparaginase from *P. vulgaris*, and also describes comparisons of some physicochemical properties of the enzyme with those of *E. coli* L-asparaginase.

Materials

Crystalline L-asparaginase (300 IU/mg of protein) was prepared by the procedure previously reported (Tosa *et al.*, 1972). DFP[1]-treated carboxypeptidase A was obtained from Sigma Chemical Co., St. Louis, Mo. PTH amino acids were obtained from Seikagaku Kogyo Co., Ltd., Tokyo, Japan. Sephadex G-25, G-150, and G-200 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Standard proteins used for molecular weight determination were obtained from Mann Research Laboratories, Inc., New York, N. Y. Guanidine hydrochloride and urea were recrystallized from 70% ethanol.

Methods

Enzyme Assay. A reaction mixture containing 20 μmol of L-asparagine and appropriate amounts of crystalline enzyme in 2 ml of 0.05 M $Na_2B_4O_7$–KH_2PO_4 buffer (pH 8.0) was incubated for 10 min at 37° and the reaction was stopped by the addition of 0.5 ml of 10% trichloroacetic acid. Liberated ammonia was determined by direct nesslerization after dilution of the reaction mixture by tenfold with water. In the presence of protein denaturants ammonia was collected with Conway's microdiffusion cuvets (Conway and Byrne, 1933).

Protein was determined spectrophotometrically based on the value of $E^{1\%,\ 1\ cm}_{280\ m\mu} = 6.6$ (Tosa *et al.*, 1972).

Ultracentrifugal Experiments. Ultracentrifugal measurements were carried out with a Beckman-Spinco ultracentrifuge Model E. For determination of sedimentation coefficient, ultracentrifugal operation was carried out at 52,640 rpm at 23.3°. The sedimentation coefficient was calculated by the method of Schachman (1957) and corrected to standard conditions.

Crystalline enzyme was dissolved in 0.1 M sodium acetate buffer (pH 6.8) and dialyzed against several changes of the same buffer at 5° for 24 hr. In the case of experiment in 3 M guanidine hydrochloride, the desalted and lyophilized enzyme was dissolved in 0.1 M sodium acetate buffer containing 3 M guanidine hydrochloride (pH 6.8) and directly analyzed without dialysis.

A sedimentation equilibrium experiment was performed by the short column method (Van Holde and Baldwin, 1958). The sedimentation equilibrium was attained after 24 hr at 5227 rpm at 20.5°. The molecular weight was estimated by

$$MW_{app} = \frac{2RT}{(1 - \bar{v}\rho)\omega^2} \frac{d \ln C}{dr^2}$$

where MW_{app} is the apparent molecular weight, R is the gas constant, T is the absolute temperature of the system, $\bar{v}$ is the partial specific volume of protein, ρ is the density of the

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[1] The abbreviations used in this paper are: DFP, diisopropyl fluorophosphate; PTH, phenylthiohydantoin.

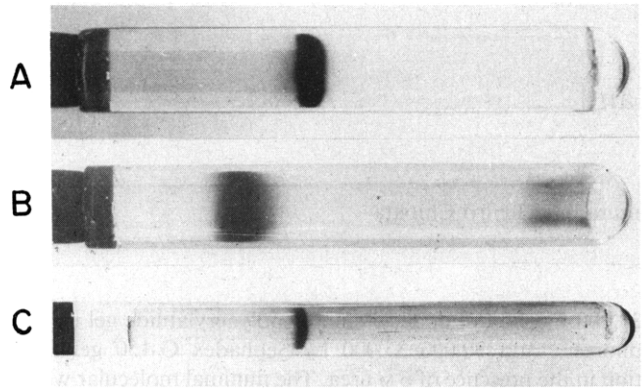

FIGURE 1: Polyacrylamide gel electrophoresis of L-asparaginase: (A) 0 M urea; (B) 7 M urea; (C) 0.1% sodium dodecyl sulfate. Conditions are given in the text.

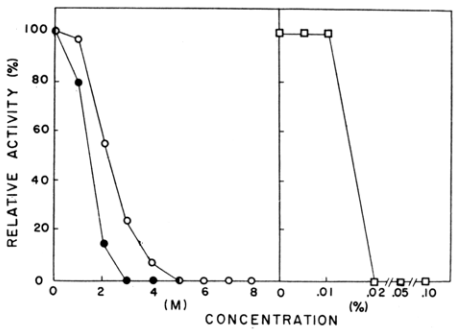

FIGURE 2: Effect of protein denaturants on L-asparaginase activity: (●) guanidine hydrochloride; (○) urea; (□) sodium dodecyl sulfate. One milliliter of enzyme solution (5 μg/ml) in 0.05 M $Na_2B_4O_7$–KH_2PO_4 buffer (pH 8.0), containing a respective concentration of agents, was preincubated for 5 min at 37°. Other conditions are given in the text.

buffer, ω is the angular velocity of rotor, and r is the distance from the axis of rotation. The partial specific volume of L-asparaginase was calculated from the amino acid composition (Isemura and Fujita, 1957), and a value of 0.74 was obtained.

Gel Filtration. Molecular weight determination by Sephadex G-150 gel filtration in the presence of 6 M urea was performed using a column (1.5 × 73 cm) equilibrated with 6 M urea–0.1 M phosphate buffer (pH 6.8). Protein samples were dissolved in the same buffer (10 mg/ml) and stood for 2 hr at 37°. One milliliter of sample solution was applied to the column and gel filtration was conducted at 7° at a flow rate of 10 ml/hr.

Disc Electrophoresis. Polyacrylamide gel electrophoresis was carried out by the method of Davis (1964) as described previously (Tosa *et al.*, 1972).

Electrophoresis in sodium dodecyl sulfate–polyacrylamide gels was performed according to Weber and Osborn (1969).

Polyacrylamide gel electrophoresis in the presence of 7 M urea was performed according to Reisfeld and Small (1966). A protein sample in electrophoresis buffer containing 7 M urea and 10% sucrose was placed on the top of spacer gel with Bromophenol Blue. Electrophoresis was conducted at 7° at 2.5 mA/tube for 2.5 hr. The gels were stained with Coomassie Blue (2.5% in 50% methanol–7% acetic acid). Destaining was performed electrophoretically in 5% methanol–7% acetic acid at 7° at 10 mA/tube for 1 hr.

Amino Acid Analysis. Crystalline enzyme was hydrolyzed according to Moore and Stein (1963) in constant boiling HCl at 110° in sealed evacuated Pyrex tubes for 24, 48, and 72 hr. Aliquots corresponding to 500 μg of protein were analyzed with a Hitachi amino acid analyzer, Model KLA-3B.

Tryptophan was determined spectrophotometrically according to Beaven and Holiday (1952) and also colorimetrically by the method of Spies and Chambers (1949).

Cystine was determined as cysteic acid after performate oxidation of sample according to Moore (1963), and also determined as carboxymethylcysteine after carboxymethylation of reduced L-asparaginase with iodoacetic acid (Clestfield *et al.*, 1963). The detection of free sulfhydryl group was performed by Ellman's reagent (Ellman, 1959) in 5 M guanidine hydrochloride.

NH_2-Terminal Amino Acid Analysis. The three-stage phenylthiohydantion method of Edman (1960) was used. Lyophilized L-asparaginase (40 mg) was subjected to phenylthiocarbamylation in 40% urea–0.15 M Tris buffer (pH 9.0–9.5) (Blömback *et al.*, 1966). Estimation of PTH amino acid was based on the absorbance at 269 mμ. PTH amino acids were identified by thin layer chromatography on silica gel (Merk, DC-Fertigplatten Kiesel Gel F_{254}) in five different solvents, *i.e.*, solvents D and E of Edman and Sjöquist (1956), solvents II and III of Brenner *et al.* (1962), and solvent V of Jeppsson (1967). The spots on the chromatograms were located by illumination with ultraviolet light and the iodine–azide reaction (Sjöquist, 1953). To differentiate between PTH leucine and PTH isoleucine, the PTH derivative was hydrolyzed with 0.1 N NaOH to the amino acid (Van Orden and Carpenter, 1964). Leucine or isoleucine was identified by thin layer chromatography in water-saturated *tert*-amyl alcohol (Consden *et al.*, 1944) and by microbioassay using *Leuconostoc mesenteroides* P-60 (Henderson and Snell, 1948).

COOH-Terminal Amino Acid Analysis. Hydrazinolysis was performed according to the procedure of Spero *et al.* (1965). Lyophilized L-asparaginase was subjected to hydrazinolysis at 100° for 10 hr. The aqueous supernatant from the benzaldehyde treatment was evaporated to dryness on a rotary evaporator at 50°. The residue was dissolved in 0.2 M citrate buffer (pH 2.2) and analyzed with the amino acid analyzer.

Carboxypeptidase A digestion was performed according to Ambler (1967). Crystalline or S-carboxymethylated L-asparaginase was allowed to react with 5% (w/w) DFP-treated carboxypeptidase A in 0.05 M Veronal buffer (pH 8.6) containing 2 M urea at 30°. Aliquots were withdrawn at appropriate times and the reaction was terminated with trichloroacetic acid (final 2%). The precipitate was removed by centrifugation and the supernatant was analyzed with the amino acid analyzer.

Results

Sedimentation Coefficient and Molecular Weight. The sedimentation behavior of crystalline L-asparaginase was studied at the protein concentrations of 0.1, 0.2, 0.4, 0.6, and 0.8% in 0.1 M sodium acetate buffer (pH 6.8) as described under Methods. The sedimentation coefficient ($s^0_{20,w}$) was calculated to be 7.9 S.

The molecular weight of crystalline L-asparaginase was determined by sedimentation equilibrium at the protein concentrations of 0.06, 0.10, and 0.14% in 0.1 M sodium acetate buffer (pH 6.8). The apparent molecular weight was calculated by the equation described in Methods. By extrapolation of the reciprocal values of apparent molecular weight to zero con-

TABLE I: Amino Acid Composition of Crystalline L-Asparaginase.

	Extrap Value per Monomer	No. of Residues (Integral) per Monomer	*E. coli* L-Asparaginase mol/33,000 g[a]
Lysine	22.8	23	21.0
Histidine	3.1	3	3.1
Arginine	6.4	6	7.2
Aspartic acid	40.0	40	50.7
Threonine	22.4	22	32.6
Serine	13.9	14	14.6
Glutamic acid	21.8	22	18.7
Proline	11.4	11	11.0
Glycine	27.3	27	28.5
Alanine	31.2	31	33.1
Valine	30.7	31	35.0
Methionine	5.2	5	3.9
Isoleucine	14.6	15	12.2
Leucine	22.6	23	22.4
Tyrosine	8.8	9	11.0
Phenylalanine	10.3	10	8.0
Half-cystine	2.0[b]		1.8
	1.9[c]	2	1.9
Tryptophan	1.2[d]		1.0
	1.3[e]	1	

[a] Ho *et al.*, 1970. [b] Determined as cysteic acid. [c] Determined as *S*-carboxymethylcysteine. [d] Determined colorimetrically. [e] Determined spectrophotometrically. Conditions are given in the text.

centration, the molecular weight of L-asparaginase was calculated to be 120,000.

Dissociation into Subunits and Their Molecular Weight. The sedimentation behavior of crystalline enzyme in 0.1 M sodium acetate buffer (pH 6.8) containing 3 M guanidine hydrochloride was studied at the protein concentration of 0.6%. The value of $s_{20,w}$ was found to be 1.6 S, indicating that the enzyme dissociates into subunits.

The molecular weight of subunits was determined by sedimentation equilibrium in the presence of 3 M guanidine hydrochloride at the protein concentrations of 0.125, 0.175, and 0.25%. The value of 29,300 was obtained, assuming that the partial specific volume was 0.74 and ρ of 3 M guanidine hydrochloride was 1.08 (Kawahara and Tanford, 1966). The determination of molecular weight of the subunit was also performed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis after reduction of the enzyme with β-mercaptoethanol (Weber and Osborn, 1969). A single protein band was observed in the gel (Figure 1), demonstrating the homogenity of subunit in molecular weight. From the calibration curve which was obtained for the mobilities of several standard proteins against the logarithm of their known molecular weight, the molecular weight of the L-asparaginase subunit was calculated to be 31,000–33,000.

Further evidence that L-asparaginase dissociates into subunits was demonstrated by gel filtration on Sephadex G-150 in the presence of 6 M urea. By comparison of elution volume of L-asparaginase to those of several standard proteins with known molecular weight, the molecular weight of subunit was found in the range of 30,000–35,000. The disulfide bridge cleaved enzyme by performate oxidation was also eluted from the Sephadex G-150 column in the same volume as the native enzyme in the presence of 6 M urea, and no smaller molecular weight daltons were found. The homogeneity of the L-asparaginase subunit was demonstrated by polyacrylamide gel electrophoresis of the enzyme in the presence of 7 M urea. As shown in Figure 1, a single protein band was found in the gel containing 7 M urea.

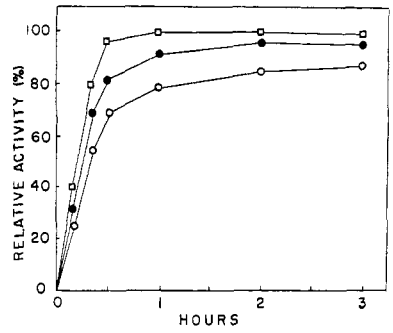

FIGURE 3: Recovery of L-asparaginase activity after dilution of protein denaturants: (●) 3 M guanidine hydrochloride; (○) 7 M urea; (□) 0.1% sodium dodecyl sulfate. The protein denaturant treatment was performed as described in the text. The protein denaturant was diluted 100-fold with 0.05 M $Na_2B_4O_7$–KH_2PO_4 buffer (pH 8.0).

Effect of Protein Denaturants on L-Asparaginase Activity. L-Asparaginase activity in the presence of protein denaturants is shown in Figure 2. Enzyme activity was completely lost in the presence of 3 M guanidine hydrochloride, 5 M urea, or 0.02% sodium dodecyl sulfate. Recovery of the enzyme activity after removal of protein denaturants was studied as follows. An enzyme solution (1 mg/ml) was treated with 3 M guanidine hydrochloride, 7 M urea, or 0.1% sodium dodecyl sulfate in 0.05 M $Na_2B_4O_7$–KH_2PO_4 buffer (pH 8.0) at 37° for 30 min, and then passed through a Sephadex G-25 column equilibrated with 0.05 M $Na_2B_4O_7$–KH_2PO_4 buffer (pH 8.0) to remove these protein denaturants. As the results show, it was demonstrated that the enzyme activity was fully recovered immediately after removal of these protein denaturants. The recovery of enzyme activity was also attained by dilution of protein denaturants. In the case of 7 M urea treatment, although the rate of recovery of the enzyme activity was somewhat slower, the enzyme activity was almost completely recovered after 1 hr of dilution of the agents at the final protein concentration of 10 μg/ml (Figure 3). The molecular weight of recovered enzyme after removal of 3 M guanidine hydrochloride was found to be 110,000–120,000 by Sephadex G-200 gel filtration. The values of 110,000–120,000 for the molecular weight of recovered enzyme are quite agreeable with the value of 120,000 for the native enzyme which was obtained by sedimentation equilibrium.

Amino Acid Composition. Amino acid composition of crystalline L-asparaginase from *P. vulgaris* is shown in Table I, and compared with that of *E. coli*. The free sulfhydryl group was not detected by Ellman's reagent even if in the presence of 5 M guanidine hydrochloride. However, one disulfide bridge per subunit was found as cysteic acid after performate oxidation or *S*-carboxymethylcysteine after reduction and carboxymethylation with iodoacetate. Since tryptophan is difficult to be determined with precision, the minimal

molecular weight was calculated by assuming one disulfide bridge per subunit, and the value of 31,400 was obtained. Carbohydrate was not detected by phenol–sulfuric acid method (Dubois *et al.*, 1956).

NH_2-Terminal Amino Acid. The NH_2-terminal amino acid of L-asparaginase from *P. vulgaris* was determined by Edman degradation. PTH leucine (0.94 mol/31,400 g) was found, and no other spots were detected on the silica gel plate in five different solvents. Since differentiation between PTH leucine and PTH isoleucine was difficult on a silica gel plate, the formation of PTH leucine was confirmed as follows. The PTH amino acid was hydrolyzed with 0.1 N NaOH to amino acid. Leucine and isoleucine were clearly identified by comparison both with the hydrolysis products of authentic PTH leucine and PTH isoleucine and also with reference leucine and isoleucine from thin layer chromatography on a silica gel plate in water-saturated *tert*-amyl alcohol. The R_F values were 0.28 for leucine and 0.23 for isoleucine. The formation of leucine from the PTH amino acid was further confirmed by microbioassay.

COOH-Terminal Amino Acid. A qualitative determination of COOH-terminal amino acid was performed by hydrazinolysis at 100° for 10 hr. Tyrosine (0.79 mol/31,400 g) was found, but no other amino acids were detected. COOH-terminal amino acid was further determined by carboxypeptidase A. Crystalline L-asparaginase or reduced and S-carboxymethylated L-asparaginase was subjected to carboxypeptidase A digestion in the presence of 2 M urea. Only tyrosine residue was quantitatively released from both COOH termini of the native and the reduced and carboxymethylated L-asparaginase (0.96 mol/31,400 g). Although the reaction was continued for 6 hr, no other amino acids were released. In the absence of urea, the COOH-terminal tyrosine residue was not released by carboxypeptidase A.

Discussion

The molecular weight of L-asparaginase from *P. vulgaris* was found to be 120,000 by sedimentation equilibrium. The enzyme dissociated into subunits of 1.6 S at the protein concentration of 0.6% in the presence of 3 M guanidine hydrochloride. The molecular weight of subunit was determined to be 29,300 by sedimentation equilibrium in the presence of 3 M guanidine hydrochloride, 31,000–33,000 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and 30,000–35,000 by Sephadex G-150 gel filtration in the presence of 6 M urea. The minimum molecular weight of L-asparaginase was calculated to be 31,400 from amino acid composition (Table I). The reliability of the value of 31,400 for the molecular weight of subunit was further confirmed by quantitative determination of terminal amino acid. One mole of leucine as the NH_2-terminal amino acid and 1 mol of tyrosine as the COOH-terminal amino acid per 31,400 g of protein were detected. These results indicate that L-asparaginase from *P. vulgaris* is composed of four subunits.

On the molecular weight of L-asparaginase of *E. coli* and its subunit, Whelan and Wriston (1969) first reported that molecular weights of 250,000, 139,000, and 64,000 were found depending on enzyme concentration. They also reported that molecular weights between 19,000 and 24,000 were found under protein denaturants, demonstrating the enzyme was composed of subunits. On the other hand, Frank *et al.* (1970) reported that L-asparaginase from *E. coli* has a molecular weight of 133,000 and is composed of four subunits of molecular weight of 33,000 by sedimentation equilibrium. They also determined the molecular weight of subunit to be 33,000 from sodium dodecyl sulfate–polyacrylamide gel electrophoresis and from amino acid composition. They could not find the evidence that the native L-asparaginase dissociates into subunits of molecular weight of 64,000 in aqueous solution. The possibility that L-asparaginase from *P. vulgaris* dissociates into subunits depending on concentrations in aqueous solution was studied by the gel filtration method. In the range of protein concentrations from 10 mg/ml to 10 μg/ml, the molecular weight of the native enzyme was found to be 110,000–120,000 by Sephadex G-200 gel filtration, and no smaller molecular weight was found. The evidence that L-asparaginase from *E. coli* (mol wt *ca.* 130,000) is composed of four subunits has been also reported from some other laboratories (Irion and Voigt, 1970; Jackson and Handschumacher, 1970; Epp *et al.*, 1971; Glossmann and Bode, 1971; Scholtan and Lie, 1971).

The identity of the subunits was suggested by Frank *et al.* (1970) from the evidence of homogeneity on polyacrylamide gel electrophoresis in 7 M urea. Greenquist and Wriston (1970) found 4 mol of leucine as the NH_2-terminal amino acid and 4 mol of tyrosine as the COOH-terminal amino acid per mole of *E. coli* L-asparaginase, indicating four equal subunits. We also found quantitatively only leucine as the NH_2-terminal amino acid and only tyrosine as the COOH-terminal amino acid of L-asparaginase from *P. vulgaris*, and found a single protein band on polyacrylamide gel electrophoresis in 7 M urea (Figure 1B). Greenquist and Wriston (1970) reported that carboxypeptidase A digestion of *E. coli* L-asparaginase resulted in the liberation of tyrosine from the COOH terminus, followed by glutamine, isoleucine, phenylalanine, and asparagine. However, we found only tyrosine from the COOH terminus and could not find the penultimate amino acid residue even when the reduced and carboxymethylated L-asparaginase from *P. vulgaris* was subjected to carboxypeptidase A digestion in the presence of 2 M urea. Arens *et al.* (1970) reported that *E. coli* L-asparaginase has isoenzymes despite homogeneity in NH_2-terminal amino acid residues (the first ten NH_2-terminal amino acid sequences were determined). They separated the isoenzymes by isoelectric focusing. However, we could not find any isoenzyme of *P. vulgaris* L-asparaginase by isoelectric focusing or chromatography on DEAE-Sephadex as previously reported (Tosa *et al.*, 1972). These results strongly suggest that the subunits of L-asparaginase from *P. vulgaris* are identical.

In the presence of 3 M guanidine hydrochloride, 7 M urea, and 0.1% sodium dodecyl sulfate, the enzyme dissociated into subunits and its activity was completely lost. However, the enzyme activity was fully recovered by removal of these protein denaturants, and the molecular weight of reconstituted enzyme was found to be 110,000–120,000 by gel filtration on Sephadex G-200. These results show that the dissociation into subunits and loss of the enzyme activity by protein denaturants are quite reversible. These results are analogous to those found by Whelan and Wriston (1969) and Frank *et al.* (1970) for L-asparaginase from *E. coli*.

Amino acid compositions of L-asparaginases from *P. vulgaris* and *E. coli* (Ho *et al.*, 1970) are very similar, except for lower contents of aspartic acid and threonine in *P. vulgaris* enzyme (Table I). One disulfide bridge per 31,400 g of *P. vulgaris* L-asparaginase is not intermolecular, but intramolecular, since the molecular weight of disulfide bridge cleaved enzyme by performate oxidation was also found in the range of 30,000–35,000 as well as the native enzyme in the presence of 6 M urea, and no smaller molecular weight was found.

In spite of similarities in some physicochemical properties

for L-asparaginases from *P. vulgaris* and *E. coli*, the immunochemical properties of the two enzymes are different (Tosa *et al.*, 1971). Phillips *et al.* (1971) reported L-asparaginase from *Serratia marcescens* cross-reacted with anti-*E. coli* L-asparaginase, but the enzymes from *Erwinia carotovora* (Wade *et al.*, 1968) and *Erwinia aroideae* (Peterson and Ciegler, 1969) were reported to be immunologically distinct from the *E. coli* enzyme. These three L-asparaginases also have antitumor activity.

Recently Cammack *et al.* (1972) reported the physical properties and subunit structure of L-asparaginase from *Erwinia carotovora*. Although the enzyme from *Erwinia carotovora* has similar physical properties as the enzyme from *E. coli*, the amino acid composition is considerably different from that of *E. coli* enzyme. *Erwinia carotovora* L-asparaginase is more basic (p*I* = 8.6), and has no disulfide bridge and tryptophan that are contained in the enzymes from *E. coli* (Arens *et al.*, 1970; Ho *et al.*, 1970) and *P. vulgaris*.

Since *Erwinia* spp., *Escherichia* spp., *Proteus* spp., and *Serratia* spp. are classified in the Enterobacteriaceae, the differences in immunochemical properties and amino acid compositions of these bacterial L-asparaginases are very interesting from the standpoint of the evolution of this enzyme.

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