Physical Properties of L-Asparaginase from *Serratia marcescens*

MALVIN L. STERN, ARTHUR W. PHILLIPS,* AND ARLAN J. GOTTLIEB

Biological Research Laboratories, Department of Biology, Syracuse University,* and Department of Medicine, Upstate Medical Center, State University of New York, Syracuse, New York 13210

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Purified L-asparaginase from *Serratia marcescens* had an apparent-weight average molecular weight of 171,000 to 180,000 as determined by electrophoresis on polyacrylamide gels and by sedimentation equilibrium at low speed in an analytical ultracentrifuge. A subunit molecular weight of 31,500 ± 1,500 was estimated for the enzyme after treatment with sodium dodecyl sulfate and urea and electrophoresis on polyacrylamide gels; a similar value was obtained by high-speed sedimentation equilibrium in the presence of guanidine hydrochloride. Our data indicate that the *Serratia* enzyme could have five or six subunits of 32,000 daltons, compared to four subunits of 32,000 daltons in the *Escherichia coli* enzyme. The *Serratia* L-asparaginase also appears to be a larger molecule than the enzyme from *Erwinia carotovora*, *Proteus vulgaris*, *Acinetobacter glutaminasificans*, and *Alcaligenes eutrophus*. The *Serratia* enzyme, like that from *E. carotovora*, was more resistant than the *E. coli* enzyme to dissociation by sodium dodecyl sulfate. This resistance could be due to the finding that the *Serratia* enzyme had a relatively high hydrophobicity, similar to the enzyme from *E. carotovora*, when compared with the hydrophobicity of the *E. coli* enzyme. The isoelectric point of the *Serratia* enzyme was approximately 5.2. The influence of certain physical characteristics of the enzyme on the biological properties is discussed.

There is a rather extensive literature on tumor-inhibitory L-asparaginases from bacterial sources, especially from *Escherichia coli* and *Erwinia carotovora*. The various physicochemical, biological, and clinical properties of L-asparaginases have been reviewed (10, 19, 54, 55). The molecular weight (MW) of the *E. coli* enzyme was reported to be 133,000 and to consist of four identical subunits of MW 33,000 (13, 16, 17, 40). Other bacterial L-asparaginases from the following species were found to be comparable to the *E. coli* enzyme in MW and subunit structure: *E. carotovora* (7, 42), *Proteus vulgaris* (50), *Acinetobacter glutaminasificans* (24, 37), and *Alcaligenes eutrophus* (J. P. Allison, J. P. Mandy, and G. B. Kitto, Fed. Proc. 30:1297, 1971).

The purification and properties of L-asparaginase from *Serratia marcescens* were previously reported (4, 5, 25, 35, 36, 38). An interesting observation, and a potentially important one in the chemotherapy of certain human neoplasms, was that considerably less *Serratia* L-asparaginase than *E. coli* enzyme was required to induce complete regression of the Gardner lymphosarcoma in the C3H mouse (4, 5). Also of interest is the observation that in non-tumor-bearing animals, the *E. coli* enzyme is cleared from the blood twice as rapidly as the *Serratia* L-asparaginase (5). The *Serratia* and *E. coli* enzymes also differ in certain of their immunological properties, although a partial cross-reactivity occurred (36). These and other observations led us to examine some of the physical properties of the *Serratia* enzyme, which are now reported.

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**MATERIALS AND METHODS**

**Bacterial strain.** Stock cultures of *Serratia marcescens* ATCC 60 were cultivated on Trypticase soy agar.

**Growth and harvesting of bacteria.** An inoculum of 100 ml, grown overnight at 37°C in a medium (21) of 4% autolyzed yeast (Nestlé Co., Inc., White Plains, N.Y.) at an initial pH of 5.0, was transferred into 10 liters of the same medium in a fermentor (New Brunswick Scientific Co., New Brunswick, N.J.) and incubated for 24 h at 34°C. Cells were harvested by centrifugation.

**Enzyme assay.** L-Asparaginase assays were performed by a method based on that of Meister (31), as described by Boyd and Phillips (4).

**Definition of enzyme unit.** One international unit
is the amount of enzyme needed to release 1.0 μmol of NH₄⁺ per min at 37°C under specified conditions.

**Protein assays.** Analyses of protein were performed spectrophotometrically as described by Layne (27).

**Purification of L-asparaginase from S. marcescens.** The enzyme was extracted from washed cells and purified to homogeneity (Fig. 1) by a modification of the method of Ho et al. (23) described by Ferguson and co-workers (14). L-Asparaginase from E. coli was a generous gift from Horace D. Brown, Merck & Co., Inc., Rahway, N.J.

**Determination of MW by disc-gel electrophoresis.** The approximate MW of L-asparaginase was determined by the analytical gel method of Hedrick and Smith (20), which consists of electrophoresis of the protein in a series of gels of increasing acrylamide concentration (Fig. 1). The relative mobility (Rm) of the protein was measured, and 100 × log (Rm × 100) was plotted against the gel concentration. The slope of this line was calculated for alkaline phosphatase, lactic dehydrogenase, and catalase as standards.

These data were then used to generate a standard curve of slope versus MW (Fig. 2). L-Asparaginase in 10% sucrose solution was loaded directly on to the gel column without the large-pore gel spacer, and MW was calculated from a standard curve (Fig. 2).

Approximate MW values of L-asparaginase were also obtained by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS). The subunits of the enzymes were first cross-linked by treatment with glutaraldehyde according to a modified procedure based upon that of Baltimore and Huang (2). One part of 33% glutaraldehyde was added to 4 parts of enzyme solution, and the mixture was kept at 4°C overnight. The electrophoretic procedures followed were those of Dunker and Rueckert (12). L-Asparaginase and the standard proteins were treated with 1% SDS, 4 M urea, and either 1% β-mercaptoethanol (BME) or 0.002 M iodoacetamide for about 1 h at 45°C. The Rm values of the proteins were calculated with the monomer of bovine serum albumin (BSA) as a marker. The Rm values for subunits of BSA, immunoglobulin, and thyroglobulin

![Fig. 1. Migration of purified L-asparaginase in polyacrylamide gel electrophoresis. Fractions (50 μl) of enzyme solution (2 mg of protein per ml) were loaded on gels containing from 6 to 10% polyacrylamide. This photograph displays the homogeneity of the enzyme preparation as well as the Rm values that are employed in the estimation of MW (see text).](http://www.asmbulletin.org/issue/0184/1110)
were plotted against their subunit MW (Fig. 3). With this standard curve and the Rm values for cross-linked L-asparaginase the MW of the latter was calculated.

**Ultracentrifugal analyses.** Sedimentation velocity and sedimentation equilibrium studies were performed in a Beckman Spinco model E analytical ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.) equipped with schlieren and interference optics and ultraviolet absorption scanning system. Sedimentation velocity measurements were also taken in a Spinco model L preparative ultracentrifuge. MW was determined by sedimentation equilibrium at high speed with meniscus depletion (8, 56) and at low speed with Rayleigh interference optics. MW values were determined at low speeds by two methods. The first method involved the use of a conventional centerpiece and column length. Meniscus protein concentrations were calculated from a separate synthetic boundary experiment (8). A second method employed the use of a modified synthetic boundary centerpiece having compartments for four different protein concentrations, which simulated the equilibrium distribution (18). However, the Nazarian analysis avoids the need for a synthetic boundary experiment in MW determinations (4, 11).

Sedimentation velocity studies were performed by both boundary analyses (8) with schlieren optics and by band analyses (39), with ultraviolet optics and an automatic photoelectric scanning system. The method of Martin and Ames (28) was also used with the Spinco model L centrifuge and sucrose gradients of 5 to 20%.

The relative hydrodynamic behavior of L-asparaginases was analyzed by the procedure described by Tanford (46). The frictional coefficients (f/f_{min}) were determined from the experimental values for s_{20,W} and the apparent-weight average MW, MW_{app}.

**Determination of the subunit MW by gel electrophoresis.** The subunit MW was estimated by the method of Swank and Munkres (45). The enzyme was dissociated into subunits in solutions containing either (i) 1% SDS and 1% BME in 0.01 M sodium phosphate (pH 7.0) or (ii) 1% SDS, 1% BME, and 8 M urea (9) in 0.01 M phosphoric acid with tris(hydroxymethyl)aminomethane (pH 6.8). Electrophoresis was performed in the presence and absence of deionized 8 M urea in the gel. The subunit MW was determined from a standard curve (51).

**Isoelectric focusing.** The isoelectric focusing of L-asparaginase was performed after the procedure of Wrigley (53) in a Buchler analytical electrophoresis apparatus with electrolytic solutions consisting of 1% (wt/vol) orthophosphoric acid in the anode compartment and 1% (wt/vol) ethanolamine in the cathode compartment.

**Amino acid analyses.** Purified L-asparaginase (about 1 mg of protein) was dialyzed against four changes of glass-distilled water in the cold for 48 h and then hydrolyzed in 6 N HCl at 110 C within sealed, evacuated glass tubes for 48 h. Amino acid analyses were carried out in the Beckman amino acid analyzer model 120C as described by Moore and Stein (33). Half-cystine was assayed as cysteic acid after performic acid oxidation (22). The statistical treatment of the amino acid data was performed by standard procedures (32, 43).

**Determination of the relative hydrophobicity of**

**FIG. 2. Estimation of the MW of L-asparaginase from S. marcescens by polyacrylamide gel electrophoresis (see text). The location of L-asparaginase on the curve is shown (x).**

**FIG. 3. Estimation of the MW of L-asparaginase from S. marcescens by polyacrylamide gel electrophoresis in the presence of SDS. All proteins (2 mg/ml) were treated for 1 h at 45 C with 1% SDS, 4 M urea and 1% BME or 0.002 M iodoacetic acid according to the method of Dunker and Rueckert (12). L-Asparaginases were first cross-linked by treatment with 1 part of 33% glutaraldehyde to 4 parts of enzyme solution. Gels containing 0.1% SDS were loaded with 50 to 100 µg of protein and subjected to electrophoresis. The estimated MW values of L-asparaginase from S. marcescens and E. coli were 177,000 (B) and 137,000 (A), respectively.
l-asparaginases. The $H_{asw}$ of l-asparaginases was calculated by the procedures described by Bigelow (3), which are based in part upon data reported by Tanford (47). This parameter is employed as a quantitative comparison of the relative stability of l-asparaginases and is derived from the amino acid analyses of the enzymes.

Chemicals and reagents. The chemicals and reagents employed are described elsewhere (4, 14).

RESULTS

Estimation of MW by polyacrylamide gel electrophoresis. The Serratia enzyme manifested one major and one minor band on polyacrylamide gels (Fig. 1). An approximate MW of 180,000 was calculated for the major band (Fig. 2). The minor band had a MW of about 330,000; it contained less than 5% of the protein in the major band and had no l-asparaginase activity. A MW of 133,000 to 135,000 for the $E$. coli enzyme was obtained with the same technique. Similar values for the MW of the $E$. coli enzyme have been reported by others (1, 16, 41).

It was possible that nonspecific aggregation occurring during electrophoresis of the enzyme could have been responsible for the relatively high MW obtained for the Serratia enzyme. We therefore treated the enzyme with glutaraldehyde to covalently link subunits. MW values of about 177,000 for Serratia enzyme and 137,000 for $E$. coli enzyme were obtained (Fig. 3) after electrophoresis of the glutaraldehyde-treated enzymes on 5% polyacrylamide gels containing 0.1% SDS.

Estimation of MW by ultracentrifugation. Values of $s_{20,w}$ for the Serratia enzyme (0.05 mg/ml) were 8.0 and 8.1 to 8.2 when measured in the analytical ultracentrifuge with schlieren and ultraviolet optics with a photoelectric scanner, respectively. In sucrose gradients (28) $s_{20,w}$ values for Serratia enzyme were 8.2 to 8.3, and for the $E$. coli enzyme, $s_{20,w}$ values were 7.8 to 7.9 (the enzyme concentration was 1.0 mg/ml).

MW determinations by sedimentation equilibrium employing the low-speed method gave MW$_{w(app)}$ values for the Serratia enzyme of 176,600 and 172,700 at 4,800 rpm and 5,600 rpm, respectively (Fig. 4A). A MW$_{w(app)}$ of 140,000 for the $E$. coli enzyme was obtained that is in the range of the values reported in the literature (16, 41). With the combined use of the Nazarian equation and the Griffith cell (11) we observed a MW$_{w(app)}$ of 179,500 for the Serratia enzyme (Fig. 4B).

Determinations of subunit MW. The approximate subunit MW$_{w(app)}$ values for the Serratia enzyme were 31,000 to 33,000 and 32,000 for the $E$. coli enzyme as measured in SDS-urea on polyacrylamide gel electrophoresis (Fig. 5). With sedimentation equilibrium ultracentrifugation in 6 M guanidine hydrochloride, a subunit MW$_{w(app)}$ of 31,300 was obtained for the Serratia enzyme (Fig. 6) with a partial specific volume of the molecule ($\bar{v}$) of 0.752 ml/g calculated from the amino acid analysis (30). $E$. coli l-asparaginase, unlike the Serratia enzyme, was completely dissociated by either 1% SDS or 1% BME. Dissociation of the Serratia enzyme was achieved only by mixtures of SDS and urea, or SDS, urea, and BME. From the amino acid analysis, a minimum subunit MW of 15,000 was calculated for the Serratia enzyme (30).

Amino acid composition. The amino acid composition of Serratia l-asparaginase is compared with that of the $E$. coli and Erwinia enzymes in Table 1. Cystine and tryptophan appear to be absent in the Erwinia enzyme, which contained a relatively large proportion of arginine. The Serratia and Erwinia enzymes manifested greater average hydrophobicities than the $E$. coli enzyme (Table 1).

Isoelectric point. The isoelectric points of Serratia and $E$. coli enzymes were observed to be 5.2 and 4.6, respectively. The Serratia preparation focused into one major band having most of the enzyme activity and a minor band with about 5% of the activity (Fig. 7). The $E$. coli enzyme separated into three major bands each of which contained active enzyme (Fig. 7).

DISCUSSION

Evidence has been presented that Serratia l-asparaginase has an approximate MW of 171,000 to 180,000 and a subunit MW of 31,500 ± 1,500 as determined by polyacrylamide gel electrophoresis and ultracentrifugal analyses. This contrasts with a MW of 133,000 to 140,000 for the $E$. coli enzyme, as observed in this as well as previous studies (1, 16, 41). The subunit MW of the Serratia enzyme appears similar to that of the enzyme from $E$. coli (16, 41), $E$. carotovora (7), and P. vulgaris (50).

The possibility that the Serratia enzyme exists as a pentamer or hexamer was suggested from our MW determinations employing gel electrophoresis and strengthened by our ultracentrifugal analyses. The nature of the data obtained by ultracentrifugal analysis merits some additional comment. Evidence has been presented that the S. marcescens enzyme preparation employed in these studies was an homogeneous preparation, and that it sedimented more rapidly than the $E$. coli and Erwinia
Fig. 4. Estimation of the MW of L-asparaginase from S. marcescens by ultracentrifugal methods. (A) Low-speed centrifugation with schlieren interference optics and a separate synthetic boundary experiment. The initial protein concentration was 0.65 mg/ml. The change in the natural logarithm (In) of the fringe number (J) is proportional to the square of the distance from the center of rotation to the specified points (radius). The initial protein concentration was 1.5 mg/ml. (B) The MW\(_{\text{app}}\) of S. marcescens L-asparaginase was estimated at 179,500 by the combined methods of Griffith (18) and Nazarian (34) as described by DiCamelli et al. (11). The initial protein concentration was 1.5 mg/ml and the rotor speed was 9,000 rpm. Symbols: (Q) constant interval through which fringe numbers are measured, (J) number of fringes in this interval.

Enzymes. The \(f/f_{\text{min}}\) values (46) indicated that the hydrodynamic behavior of the S. marcescens enzyme \(f/f_{\text{min}} = 1.36\) differed from that of a spherical molecule to a greater extent than did the E. coli enzyme \(f/f_{\text{min}} = 1.17\). These results suggest that these two enzymes differ in either the number, or the spatial arrangement, of their subunits.

The MW\(_{\text{app}}\) of homogeneous *Serratia* enzyme was 172,600 to 176,700 as determined by low-speed sedimentation equilibrium. These values were in good agreement with data obtained from gel electrophoresis and are consistent with a pentameric or more likely a hexameric structure. In other similar experiments at both low speed (3,200 to 6,000 rpm) and high speed (12,000 to 14,000 rpm) a time-dependent, irreversible, partial dissociation of the *Serratia* enzymes.
enzyme during prolonged ultracentrifugation was observed similar to that reported by Hol- cenberg et al. (24) for the glutaminase-asparaginase from A. glutaminasificans. When \( MW_{w,a} \) values (50,000 to 140,000) were calculated in these studies at various rotor speeds and plotted against their \( \Delta J \) values (J is the fringe number), the \( MW_{w,a} \) decreased with protein concentration and varied with rotor speed. Both phenomena are characteristic of protein dissociation (39, 49). Since the lowest MW found was greater than that of the monomer (50,000 versus 32,000), it appeared that dissociation was not complete. After centrifugation only 70% of the enzyme activity was retained, indicating the possibility of a partial, irreversible dissociation. Finally, the dissociation was shown to be dependent upon the time required to reach equilibrium in the centrifuge. When the time required to reach equilibrium was decreased from 48 to 13 h, no dissociation occurred, and the MW values that were obtained for a "homogeneous" protein were in close agreement with those obtained from gel electrophoresis.

The subunit MW of the Serratia and E. coli enzymes obtained from gels approximated our values from analytical ultracentrifugation, and our values for the E. coli enzyme compare well with previously reported values (16, 17, 40). However, there may be some doubt about our subunit MW values from ultracentrifuge data since our \( \tilde{v} \) value was determined from amino acid analyses only, and the \( \tilde{v} \) of proteins can be altered in guanidine hydrochloride due to hydration and solute-solvent interactions (48).

### Table 1. Relative hydrophobicities of L-asparaginases from S. marcescens, E. carotovora, and E. coli

<table>
<thead>
<tr>
<th>Amino acid side chain hydrophobicity ( \text{value} \xrightarrow{(\text{joules})} ) (joules/residue)</th>
<th>Relative hydrophobicity ( \text{value} \xrightarrow{(\text{g of protein})} \times 10^3 )</th>
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<tbody>
<tr>
<td>Asp</td>
<td>39</td>
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<tr>
<td>Thr</td>
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<tr>
<td>Ser</td>
<td>10</td>
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<td>Glu</td>
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<td>Arg</td>
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\(*\) Values reported by Tanford (47). The values of 0 joules/residue are employed as suggested by Bigelow (3).

\(\tilde{v}\) The average hydrophobicities of Serratia, Erwinia, and E. coli L-asparaginases were calculated to be 1.035, 1.055, and 0.998 joules/residue, respectively. Average hydrophobicity was calculated by dividing the total hydrophobicity by the total number of amino acid residues present (3).

\(\dagger\) Based upon data from Cammack et al. (7).

\(\ddagger\) Calculated from values reported by Ho et al. (23).

\(\star\) Value taken from Wriston and Yellin (55).
Erwinia, and E. coli asparaginases in aqueous solution was examined from the viewpoint of hydrophobicity, which is important for the stability of proteins in the presence of denaturants (3, 6, 47). An examination of the data indicates a correlation between hydrophobicity and dissociation characteristics of the enzymes. For instance, the Erwinia enzyme was resistant to complete dissociation in 8 M urea and 1% SDS (7), and the Serratia enzyme was slightly less resistant in that 8 M urea, but not 1% SDS, completely dissociated the enzyme. On the other hand, the E. coli enzyme was completely dissociated by 0.1% SDS under mild conditions. These differences are reflected in the following average hydrophobicities (3) of the enzymes: 1.055, 1.035, and 0.998 kcal/residue were calculated for the enzyme from Erwinia, Serratia and E. coli, respectively (Table 1). The importance of hydrophobic interactions to the dissociation of L-asparaginase has been discussed by Cammack et al. (7). These investigators suggested that the interaction binding the subunits of Erwinia enzyme was hydrophobic in nature because optical rotatory dispersion spectra indicated an apparent increase in helix content of the subunits released by SDS. This absence of polypeptide unfolding with dissociation was taken to indicate that hydrophobic interactions existed between subunits. The calculated hydrophobicities for the enzymes suggest that the subunits of Serratia and Erwinia enzymes are bound together in a similar fashion. In the Erwinia enzyme, disulfide bonds are not involved since no half-cystine residues were observed (7). The Serratia enzyme was not completely dissociated by 1% BME but contained three half-cystine residues per subunit.

Data obtained from isoelectric focusing indicate that the Serratia and E. coli enzymes are acidic proteins (23, 29, 52), whereas the Erwinia enzyme is a basic protein (7). The isoelectric point we observed for the Serratia enzyme differs from that of the E. coli enzyme. This difference may not be significant in view of the findings of Laboureur et al. (26), who reported the appearance of species of E. coli enzyme of increasing electronegativity during purification and storage. The change observed was temperature dependent and it occurred at low temperatures. These investigators suggested that the changes were nonenzymatic and were analogous to alterations in cytochrome c in which the conversion of subfraction cy I occurs successively to cy II, cy III, and cy IV with the release of sufficient ammonia for the hydrolysis of one amide group per conversion (15). This might also explain why Mashburn and Landin (29)

**Fig. 7.** Isoelectric focusing of L-asparaginase from S. marcescens and E. coli. The S. marcescens enzyme (A) displayed one major band and one minor band each containing enzyme activity whereas the E. coli enzyme (B) showed three intense, enzymatically active bands. The isoelectric points were 5.2 and 4.6 for the S. marcescens and E. coli enzymes, respectively.

Tanford et al. (48) found that the $\tilde{v}$ of a protein in 6 M guanidine hydrochloride was 0.01 ml/g less than the $\tilde{v}$ in dilute aqueous salt solutions.

The differential stability of the Serratia,
reported a range of isoelectric points (4.6 to 5.5) for the E. coli asparaginase. The findings of Laboureur et al. could also explain our observation of three to four bands containing active enzyme during isoelectric focusing of the E. coli enzyme. The same explanation could be invoked relative to the difference in isoelectric points between the Serratia and E. coli enzymes and the presence of a minor, enzymatically active band during isoelectric focusing of the Serratia enzyme.

In conclusion, evidence has been presented that L-asparaginase from Serratia is a somewhat larger molecule than the enzyme from E. coli and certain other bacteria; furthermore, the Serratia enzyme may exist as a pentamer or, more likely, a hexamer rather than the usual tetrameric form of bacterial asparaginasins. The Serratia enzyme also possesses more hydrophobicity and is thus more resistant to certain denaturants than the E. coli asparaginase. The Serratia and Erwinia enzymes are similar in these latter respects. The increased size and hydrophobicity of the Serratia enzyme compared with E. coli L-asparaginase possibly could account for the decreased blood clearance rate of the Serratia enzyme in the C5H mouse (5). This in turn might explain the relatively greater efficiency of the S. marcescens enzyme in the regression of the 6C3HED lymphoma in the C3H mouse (4, 5).

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LITERATURE CITED


