The N, O-DiacetylMuramidase of Chalaropsis Species

II. PHYSICAL PROPERTIES*

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SUMMARY

Lysozyme Chalaropsis is a single polypeptide chain with an apparent weight average molecular weight of 19,100 ± 900. The ultraviolet absorption is typical for proteins with an $E_{1	ext{cm}}$ = 24.8 ± 0.4, and its isoelectric point is weakly alkaline at 7.53. The sedimentation constant ($s_{20,w}$) of 2.27 ± 0.14 exhibits no apparent concentration dependence, a phenomenon consistent with a symmetrical sedimenting mass. The secondary structure, as judged by optical rotatory dispersion in the ultraviolet, exhibits significant structural differences as compared to hen egg white lysozyme.

The specificity of a fungal Chalaropsis species, crystalline bacteriolytic enzyme has been established recently as a β-1,4-N-acetylmuramidase (1), a specificity identical with hen egg white lysozyme. However, the Chalaropsis lysozyme (henceforth designated lysozyme Ch) also exhibits β-1,4-N, O-diacetylmuramidase activity, in contrast to egg white lysozyme. This specificity difference offers a unique opportunity for comparative enzyme structure analysis. A physical characterization of lysozyme Ch is described in this communication.

EXPERIMENTAL PROCEDURES

Lysozyme Ch—Twice crystallized lysozyme Ch prepared as previously described (1) was used in these studies.

Extinction Coefficient.—The absorption spectrum of lysozyme Ch was determined in a Cary model 14 recording spectrophotometer in both distilled water and 0.1 M acetate buffer, pH 5.0. The spectra were identical, with typical protein absorption maxima at 280 μ. Extinction coefficients were determined on samples of enzyme in distilled water. Absorbance measurements were made on a series of protein concentrations in a Beckman DU spectrophotometer at 280 μ and aliquots were dried in small glass cups to constant weight in a drying pistol (refluxing toluene, over P₂O₅ and under vacuum). Dry weights were determined on a Mettler balance with an accuracy of ±10 μg. Over the range 0.05 to 1.2 a linear relationship exists between absorbance and dry weight. Concentrations of protein solutions in these studies were determined with the experimentally determined extinction coefficient in 0.1 M acetate buffer, pH 5.0.

Sedimentation Studies.—Sedimentation velocity and sedimentation equilibrium studies were performed on a Spinco model E ultracentrifuge equipped with schlieren and interference optical systems. All sedimentation velocity experiments were performed at 20° in double sector cells and the observed sedimentation coefficients were corrected to values corresponding to water at 20° ($s_{20,w}$). The value of $s_{20,w}$ reported was obtained by extrapolation to infinite dilution.

Both high (2) and low speed (3) sedimentation equilibrium experiments were made at 20° with 3-mm columns layered over FC 43 (perfluorotributylamine, Minnesota Mining and Manufacturing Company). The initial concentrations ($C_o$) in fringes were established with a synthetic boundary cell for the low speed equilibrium runs with the weight average molecular weight ($M_w$) for the entire liquid column determined by the relationship:

$$M_w = \frac{2 RT}{(1 - \bar{V} \rho) a^2 b^2} \frac{1}{C_{eq}}$$

in which $K$ is the gas constant, $T$ the absolute temperature, $\bar{V}$ the partial specific volume, $\rho$ the buffer density, $\omega$ the angular velocity, $b$ the base and $a$ the meniscus of the liquid column, and $C_{eq}$ the concentration gradient in fringes between $b$ and $a$ at equilibrium. Meniscus and base $M_w$ values were determined by plots of ln against $x^2$ (see high speed methodology) in which the concentration at the middle of the liquid column ($C_i$) was evaluated by the relationship:

$$C_i = \frac{C_{eq}(\sigma^2 - a^2)}{\epsilon (\sigma^2 - b^2)}$$

where

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where $A = M \left(1 - \frac{V}{V_p} \right) \omega^2 / 2 RT$. Least squares analyses over the whole column gave close agreement with Equation 1.

High speed equilibrium experiments deplete the meniscus of protein, eliminating the necessity of separate synthetic boundary runs, and were evaluated by the relationship:

$$M_\infty = \frac{2RT}{(1 - \frac{V}{V_p})\omega^2} \frac{d \ln c}{dz^2}$$  \hspace{1cm} (3)

where $z$ is the distance from the center of rotation. The Rayleigh interference system was used for all determinations with fringe positions recorded on Spectroscopic II G photographic plates (Kodak) and analyzed with a Nikon model 6 micro comparator at 50X magnification. A value of 5.62 cm was used as the position of the counterbalance wire from the center of rotation. Extensive dialysis was used to ensure equilibration of solvent and solution. Runs were made for 20 to 24 hours, equilibrium being assured by a lack of change in fringe concentration gradient on sequential photographs.

Z-average molecular weight ($M_z$) for the low speed runs were calculated from the $M_\infty (b)$ and $M_\infty (a)$ obtained from the base and meniscus slopes, respectively, of the ln c against $z^2$ plots, by the relationship:

$$M_z = \frac{M_\infty (b) C_b - M_\infty (a) C_a}{C_a}$$  \hspace{1cm} (4)

where $C_b$ and $C_a$ are the fringe concentrations at the base and meniscus.

The type of aggregation phenomena (dimer as opposed to higher order aggregate) observed in the low speed sedimentation experiments was evaluated by the following expression which relates $M_z$ to the content of dimer in a monomer-dimer equilibrium:

$$M_z = \frac{1 + 3 \frac{C_2}{C_1 + C_2}}{1 + \frac{C_2}{C_1 + C_2}}$$  \hspace{1cm} (5)

in which $M_z$ in this case was taken as $M_\infty (a)$ and $C_1$ and $C_2$ are the concentrations of monomer and dimer, respectively.

A single high speed experiment was made in guanidine-HCl to establish whether the experimentally determined $M_\infty$ was the monomeric weight. Guanidine-HCl was recrystallized from methanol after decolorizing with Norite. The enzyme was reduced with dithiothreitol and extensively dialyzed against 5 M guanidine-HCl containing 1 mM dithiothreitol. There is still some degree of uncertainty regarding the partial specific volume of proteins in guanidine-HCl. Values ranging from no apparent change (4) to a 1 to 2% decrease in the apparent partial specific volume (5) have been reported. Nevertheless, the results in this case allow a clear assessment of whether or not there is subunit structure.

**Solution Densities and Partial Specific Volumes**—Leach pycnometers with a volume of 50 ml were used at 20°C for all density determinations. The apparent partial specific volume ($V_{app}$) was determined in water.

**Isoelectric Point**—The isoelectric point of lysozyme Ch was determined by the isoelectric focusing technique (6). The temperature was maintained at 20°C ± 0.5 by a Tamson circulating water bath. Two separate experiments were performed with a pH gradient of 3 to 10 in one case and 5 to 8 in another, and a protein concentration of 4 mg per run. Measurements of pH were performed with a Radiometer pHM4d meter and a combined electrode calibrated to ±0.01 pH unit. Activities were measured by the lysis of cell-free staphylococcal cell wall suspensions (7).

**Optical Rotatory Dispersion**—Measurements of the optical rotatory dispersion from 350 rpm to 210 rpm for lysozyme Ch were made on a Cary model 60 recording spectropolarimeter. Experiments were carried out at 27°C with a 1-cm path length cell in both sodium phosphate (pH 6.5, 0.1 M) and water at 0.005% protein concentration with two different preparations with equivalent results. Experimental data are reported as the mean specific rotation $[\theta]_\lambda$ based on the relationship:

$$[\theta]_\lambda = \frac{MRW}{100} \frac{3}{(n^2 + 2)} |\phi_\lambda|$$  \hspace{1cm} (6)

where $MRW$ (mean residue weight) was taken as 104, $n$ (the refractive index) of phosphate buffer as 1.3346, and $|\phi_\lambda|$ the specific rotation at wave length $\lambda$. Lysozyme Ch was analyzed with respect to Cotton minima and one-term Drude (8) and Moffitt parameters (9). A similar analysis was made for hen egg white lysozyme for comparative purposes.

**Statistical Methods**—All linear regression lines were calculated by the method of least squares with a Wang 360 calculator and card programer (Cal 360-STAT-6 program), and ungrouped data were analyzed with the Cal 360-STAT-1 program. Statistically analyzed results are reported as ±1 S.D.

**RESULTS**

**Extinction Coefficient**

A value for $E_{280}^\text{em}$ was determined to be 24.8 ± 0.4. This constant has been confirmed by fringe counts in the ultracentrifuge.

1 J. W. Shih and J. H. Hash, unpublished observations.
2 Wang Program Library, Vol. 1, Wang Laboratories, Inc., Tewksbury, Massachusetts.
CONCENTRATION (mg/ml)

**Fig. 2.** Sedimentation velocity data for lysozyme Ch. $s_{20,w}$ evaluated at infinite dilution.

**Fig. 3.** Typical low speed sedimentation equilibrium $\ln c$ against $x^2$ plot of fringe concentration gradient at equilibrium for lysozyme Ch (Run 1, Table I).

**TABLE I**

| Run no. and buffer (0.1 M) | Average speed | Concentration % | $M_w$ | $M_c$ |
|----------------------------|---------------|-----------------|-------|-------|
|                            |               | Whole column, Meniscus, Base, $M_c$ |       |       |       |
| 1. Sodium acetate, pH 5.0  | 16,150        | 0.25, 19,350, 17,700 | 20,530 | 21,490 |
| 2. Sodium acetate, pH 5.0  | 16,150        | 0.17, 19,900, 18,200 | 20,370 | 21,090 |
| 3. Sodium acetate, pH 5.0  | 16,150        | 0.09, 20,500, 18,700 | 20,940 | 21,900 |
| 4. Potassium phosphate, pH 6.5 | 16,150 | 0.60, 20,000, 18,900 | 21,320 | 22,150 |
| 5. Potassium phosphate, pH 6.5 | 16,150 | 0.39, 19,800, 18,380 | 20,640 | 21,510 |
| 6. Potassium phosphate, pH 6.5 | 16,150 | 0.40, 20,000, 18,550 | 20,580 | 21,210 |
| 7. Potassium phosphate, pH 6.5 | 16,150 | 0.30, 19,800, 18,000 | 20,480 | 21,140 |
| 8. Potassium phosphate, pH 6.5 | 16,150 | 0.21, 19,200, 17,600 | 19,730 | 20,470 |

Average $M_w$:

- **Calculated by Equation 1.**
- **Calculated by Equations 2 and 3.**
- **Calculated by Equation 4.**
from four separate runs (Table II) cluster around a \( M_w = 20,000 \pm 700 \).

A single experiment with lysozyme Ch in 5 M guanidine-HCl with the enzyme sulfhydryls in the reduced state revealed a \( M_{w,app} = 20,000 \), a value indicative of a single polypeptide chain for the native enzyme.

**Isoelectric Point**—Two determinations of the isoelectric point with the use of the technique of isoelectric focusing yielded values differing by only 0.01 pH unit. Sharp, symmetrical boundaries were obtained with this technique as indicated by Fig. 5. The observed isoelectric point was 7.53 at 20°.

**Optical Rotatory Dispersion**—The optical rotatory dispersion of lysozyme Ch reveals significant differences in secondary structure as compared to egg white lysozyme (Fig. 6). The latter possesses the typical Cotton minima at 223 mp (\([\eta]_m\) of \(-3400^\circ\)), a feature common to the majority of globular proteins. Treatment of the data for egg white lysozyme by the Moffitt equation and a \( \lambda_0 \) of 212 yielded an excellent linear correlation and a \( b_0 \) of \(-145 \pm 8 \) in close agreement with the data of Tomimatsu and Gaffield (11). Lysozyme Ch exhibits a Cotton minima of \(-6400^\circ\) for \([\eta]_m\) at 227 mp. A one-term Drude plot revealed an excellent linear correlation with \( \lambda_0 = 218 \) mp as opposed to 245 mp for egg white lysozyme. The data as sub-

\[ \text{Fig. 4. Typical high speed sedimentation equilibrium data for lysozyme Ch (Run 4, Table II).} \]

\[ \text{Fig. 5. Isoelectric focusing of lysozyme Ch over the pH ranges 3 to 10 (upper graph) and 5 to 8 (lower graph). Solid line represents pH gradient, evaluated on left ordinate, and dashed line represents enzymatic activity, evaluated on right ordinate. Upper plot, 6.7 ml per fraction; lower plot, 4.7 ml per fraction.} \]

\[ \text{Fig. 6. The optical rotatory dispersion of lysozyme Ch and egg white lysozyme.} \]

| Run no. and buffer \( (0.1 \text{ M}) \) | Average speed | Concentration \( % \) | \( M_w \) |
|----------------------------------------|--------------|-----------------|-------|
| 1. Potassium phosphate, pH 6.5 .......... | 47,200       | 0.05            | 19,800 |
| 2. Sodium acetate, pH 5.0 ............. | 47,350       | 0.08            | 19,150 |
| 3. Sodium acetate, pH 5.0 ............. | 47,400       | 0.05            | 20,000 |
| 4. Sodium acetate, pH 5.0 ............. | 47,350       | 0.025           | 21,100 |
jetted to the Moffitt treatment, with λ0 = 212, 216, and 220 mµ, gave the best linear correlation with λ0 = 220 mµ for lysozyme Ch. The b0 in this case was +195 ± 135 with an a0 = −1014 ± 48. The ultraviolet shift of the Cotton minima and the small positive b0 value are unusual optical features. However, these optical rotatory parameters are quite similar to a small group of globular proteins reported by Jirgensons (12).

DISCUSSION

The physical properties of lysozyme Ch examined in this study and contrasted to hen egg white lysozyme are summarized in Table III. It is evident that the molecular structure of this fungal muramidase shows considerable differences from the well known egg white lysozyme. The apparent molecular weight of lysozyme Ch is approximately one-third higher than the established value for egg white lysozyme. The previous demonstration of a high degree of homogeneity of these lysozyme Ch preparations (1) indicates that the high molecular weight material observed in low speed equilibrium experiments (Fig. 3) is most likely the result of a small amount of aggregation. However, since the value cited is a weight average molecular weight, the chemical weight may be slightly reduced. The lack of concentration dependence in the sedimentation velocity experiments is indicative of a spherical sedimenting mass. As compared to hen egg white lysozyme there is a large difference in isoelectric point, a property related to the primary structure. Other differences in these two muramidases may also be noted by the apparent alterations in secondary structure as observed with optical rotatory dispersion studies. The observed λ0 falls within the range commonly associated with denatured proteins (15). In addition, as compared to egg white lysozyme, lysozyme Ch exhibits a shift in its Cotton minima from 233 mµ to 227 mµ and a low or positive b0, a feature suggested by Jirgensons to be applicable to the presence of β structure (12). Although nonprotein moieties may be responsible for the optical rotatory behavior, preliminary chemical studies indicate that no carbohydrate is present.

Table III

| Comparative physical properties of lysozyme Ch and egg white lysozyme |
|---------------------------------------------------------------|
| Subunit structure          | Lysozyme Ch | Egg white lysozyme        |
| Molecular weight           | Single chain | Single chain              |
| Sedimentation coefficient  | 19,100 ± 900 | 14,400 ± 100               |
| Partial specific volume (V)| 2.27 ± 0.14 | 1.91                      |
| Isoelectric point          | 0.726 ml/g  | 0.705 ml/g                |
| Extinction coefficient     | 7.53        | 11.1                      |
| Optical rotatory dispersion| 24.8 ± 0.4  | 26.35 ± 0.15               |
| Cotton minima             | 227 mµ      | 233 mµ                    |
| λ0 (Drude, one term)       | 218 mµ      | 245 mµ                    |
| b0 (Moffitt)               | +195 ± 135  | −145 ± 8                  |
| λ0 (best fit of data in Moffitt plot) | 220 mµ | 212 mµ                     |

a Molecular weight evaluated from average meniscus value of low speed sedimentation equilibrium and average value of high speed sedimentation equilibrium data.

b Data from Sophianopoulos et al. (13).

c Data from Tanford (14).

d Data from this study. For comparison to literature see Tomimatsu and Gaffield (11).

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