Confirmation of Molecular Weight of *Aspergillus oryzae* α-Amylase Using the Low Angle Laser Light Scattering Technique in Combination with High Pressure Silica Gel Chromatography

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Molecular weight of *Aspergillus oryzae* α-amylase (Taka-amylase A) was estimated to be 51,000 ± 500 by the combined use of high pressure silica gel (TSK-GEL G3000SW) chromatography and the low angle laser light scattering technique. The study was carried out partly to assess the performance of the combined technique, and results obtained indicate that it is highly promising as a method to determined protein molecular weight both accurately and quickly.

The *Aspergillus oryzae* α-amylase [EC 3.2.1.1], often called “Taka-amylase A,” has been much studied in Japan (1). High resolution X-ray crystallographic study of the enzyme is now being carried out, and 0.3 nm resolution crystal structure has been reported by Matsuura et al. (2). Knowledge of the number of amino acid residues of the enzyme molecule is a prerequisite to any attempt to trace the arrangement of the polypeptide as a three-dimensional electron density map but its amino acid sequence has not been worked out, although the number can be deduced from the molecular weight of the α-amylase. However molecular weight of the α-amylase has been reported variously ranging between 49,000 and 54,000 (for references, see Ref’s. 1 and 2).

The present study was planned to confirm the molecular weight of the α-amylase. Molecular weight of a protein is liable to be estimated incorrectly due to changes in the molecular species during the preparation and measurement of a sample; these may be association or degradation due to disulfide exchange or proteolysis by proteases contained as contaminants. Presence of one sulfhydryl and four disulfide groups in the α-amylase molecule and the abundance of proteases in the source of the enzyme suggest that such changes are likely to be responsible for the variety of the reported molecular weight (4).

Accordingly we planned to estimate the molecular weight of the *A. oryzae* α-amylase by a method which requires far less time in preparation and measurement. It is a combination of the two recently developed techniques, high pressure silica gel chromatography using a column of TSK-GEL G3000SW (4, 5) and the low angle laser light scattering technique (6). The practicability of the technique has been shown by Fukuda et al. (7). The present study was carried out primarily to confirm the molecular weight of the α-amylase, but also to make a further assessment of the technique.

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MATERIALS AND METHODS

The *A. oryzae* α-amylase was extracted from Takadiastase Sankyo and purified essentially according to the procedure described by Toda and Akabori (8). Another preparation of the α-amylase identical to that used in the X-ray study (2) was donated by the X-ray crystallographic group of our Institute. Bovine serum albumin, hen's ovalbumin, and bovine pancreatic ribonuclease A were highly purified crystalline preparations, and obtained from Armour, Prof. S. Takagi, and Sigma, respectively. Dextrans T-20, T-70, and T-500 (nominal weight-averaged molecular weights; 22,300, 70,000, and 478,000, respectively) were purchased from Pharmacia.

In preliminary experiments, 0.05 M sodium phosphate buffer, pH 7.0, containing 0.02% sodium azide was used. The molecular weight of the α-amylase was measured in 0.01 M sodium acetate of pH 6 containing 0.2 M sodium chloride and 0.02% sodium azide. Phosphate buffer was not used, because it deprives calcium of the amylase to destabilize it and to form precipitates of calcium phosphate.

Light scattering was measured with a low angle laser (He-Ne, 633 nm) light scattering photometer, model LS-8, of Toyo Soda Co., Ltd. with a flow-through cell with an internal volume of 30 µl. Output of the photometer was recorded on a dual-pen recorder together with that of a refractometer. All measurements were made at room temperature, 25±1°C. Batch-type measurements were carried out using a home-made sample injection system described elsewhere (9). Sample proteins were made in equilibrium with the buffer used and free from unfavorable aggregates by gel filtration through a column of Sepharyl S-300 (2.5 x 80 cm). In gel chromatography-type experiments, samples were applied to the scattering photometer via a silica gel column, and then to a precision differential refractometer (For details, see Fig. 1).

Specific refractive index increments were measured with a precision differential refractometer, model RM-102 of Union Giken Co. at 633 nm and at 25±0.03°C. Protein samples were thoroughly dialyzed against the buffers used in concentration around 10 mg/ml. Protein concentration was determined by the dry weight method.

RESULTS

The output of the low angle laser light scattering photometer can be correlated with the molecular weight of a sample protein as follows (9),

\[
\frac{n_0^2 (\frac{dn}{dc})^k c}{(Output)_{LS}} = \frac{1}{M} + 2Bc
\]

where \(n_0\), \(dn/dc\), \(c\), \(M\), and \(B\) are the refractive index of the solvent at 633 nm, the refractive index increment of the protein at 633 nm, the weight concentration of the sample protein, the molecular weight of the protein, and the second virial coefficient, respectively. The constant, \(k\), is an instrument constant which can be determined from
measurements using standard polymers of known molecular weights.

Figure 2 shows the plots of the left side of Eq. 1 versus protein concentration for three kinds of proteins. In this case, the constant, $k$, was estimated to be 6.8 x 10^{-4} mg·ml^{-1}·mV (average of the values estimated using Dextran T-20, T-70, and T-500 to be 6.7, 7.1, and 6.5 x 10^{-4}, respectively). The values of $dn/dc$ of the three Dextrans were assumed to be 0.147 ml/g (10). The values for bovine serum albumin and ovalbumin were determined to be 0.191 and 0.184 ml/g, respectively. Ribonuclease A was assumed to have the same value of $dn/dc$ as that of bovine serum albumin. Molecular weights of bovine serum albumin, ovalbumin, and ribonuclease A were therefore estimated to be 69,000 (65,400 (11)), 44,000 (42,700 (12)), and 14,000 (13,800 (13)), respectively. These values are in good agreement with those obtained by the sequence determination of amino acid residues shown in the parenthesis. The good performance of the light scattering photometer was thus confirmed, and further measurements were performed in a simpler mode as will be described below.

Elution of sample proteins from a column of TSK-GEL G3000SW was monitored by measurement of the changes in light scattering and refractive index using the low angle laser light scattering photometer and the precision differential refractometer, respectively. A typical example of the records is shown in Fig. 3. The arrows on the left indicate a sample injection. The sample was bovine serum albumin dissolved in the elution solvent (0.01 M sodium acetate of pH 6 containing 0.2 M NaCl and 0.02% NaN₃) to a final concentration of 1.0 mg/ml, and loaded beforehand in the sample loop with an internal volume of 200 μl. The changes in the intensity of light scattering recorded at the bottom appear ahead of the corresponding ones in the refractive index, because of the differences in the positions of the recorder pens and the cells of the detectors along the flow-line.

"Peak 1" in the record of light scattering seems to correspond to highly aggregated albumin eluted at the void volume. They are negligibly small in quantity as is clear from the absence of a corresponding peak in the record of refractive index. "Peak 2" in the bottom curve may be assigned to a mixture of oligomers of albumin, and has a counterpart, "shoulder 2." "Peak 3" and the "peak 3'" may both be safely assigned to the dimer of albumin, because the intensity ratio between them indicates that the component has a molecular weight twice of that of the mono-

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**Fig. 2.** Determination of molecular weights of the three kinds of proteins by low angle laser light scattering photometer by the batch-type experiments. From top to bottom; ribonuclease A, ovalbumin, and bovine serum albumin. See text for the results obtained.

**Fig. 3.** Elution patterns of bovine serum albumin from the TSK-GEL column detected by measuring changes in refractive index (top) and light scattering (bottom). Sample, 0.20 mg of bovine serum albumin in 200 μl of the elution buffer; chart speed, 30 cm/h; pressure, 30 kg/cm²; flow rate, 0.70 ml/min. Gain setting: LS-8, 16; RI-8, 128. For details, see text.
meric albumin. The component detected as "peak 4" and "peak 4'" is the major component, and can be assigned to the monomeric bovine serum albumin. The trough in the upper curve indicated by the asterisk corresponds to the elution of the solvent of the sample which usually differs slightly from the elution solvent with respect to refractive index.

Ovalbumin and the A. oryzae α-amylase were also applied to the measuring system shown in Fig. 1, and gave the elution patterns shown in Fig. 4. Presence of oligomers was observed with ovalbumin (Fig. 4A). Our preparation of the amylase was found to be free from this component (Fig. 4B), and the one used for X-ray analysis also gave a similar pair of elution patterns.

The elution patterns of the major peaks in Figs. 3 and 4 indicate that the initial concentration decreases one fourth or more during its passage through the column at the positions with maximum concentration. Experiments made to draw lines a and a' and line b in Fig. 5 were carried out using sample solutions with protein concentration of 1 mg/ml and 2 mg/ml, respectively. The batch-type experiments shown in Fig. 2 indicate that the dependence of scattering intensity on protein concentration is negligible when it is below 1 mg/ml. The second term of Eq. 1, thus, can be ignored in the gel chromatography-type experiments.

The output of the refractometer, \((Output)_{RI}\), is nominally said to be proportional to the difference in refractive index between the solution and the solvent. If so, the concentration, \(c\), in Eq. 1 can be replaced by \((Output)_{RI}/(dn/dc)\) with the addition of a dash to the constant, \(k\). Ignoring the second term in Eq. 1, this gives Eq. 2 as:

\[
\frac{\frac{Output}{}_{LS}}{(Output)_{RI}} = n_0^* \left(\frac{dn}{dc}\right) k'M
\]  

Strictly speaking, the differential refractometer monitoring the elution must use a light source with the same wavelength as that of the refractometer used to measure \(dn/dc\). The former is actually using a white light. The ratios of the output of the former to that of the latter, therefore, is not necessarily constant among proteins. For the three proteins used in the present chromatography-type experiments, the ratios were, however, found to agree with an error of 2% or less and thus allow the use of Eq. 2 in the present study.

Refractive index increments were estimated to be 0.191, 0.185, and 0.184 ml/g for bovine serum albumin, the α-amylase, and ovalbumin, respectively. Values \((dn/dc)M\) on the right of Eq. 2 were plotted versus the output ratios for the standard proteins, bovine serum albumin and ovalbumin, in Fig. 5. The molecular weight of the α-amylase was estimated by interpolation as shown in Fig. 5 for two series of experiments. Line a was obtained using our preparation of the α-amylase. The ratios in the abscissa were estimated from the height of the peaks typical examples of which are shown in Figs. 3 and 4. The two arrows indicate the range of the mean deviation obtained in three experiments. Molecular weight of the α-amylase was estimated by interpolation as shown in Fig. 5 for two series of experiments.

Fig. 4. Elution patterns of ovalbumin (A) and the A. oryzae α-amylase (B) obtained in the same manner as in Fig. 3. Sample: A, 0.2 mg; B, 0.14 mg. Only records made 10 min after injection are reproduced.

Fig. 5. Calibration lines used to determine molecular weight of the A. oryzae α-amylase. For details, see text.
weight of the α-amylase was thus estimated to be 51,000 ± 500.

Line $a'$ in Fig. 5 shows the corresponding plot where the intensities were estimated from the areas of the peaks. Judging from the mean deviation, the former way of estimation of the intensity ratios is superior to the latter. Disagreement of lines $a$ and $a'$ may be ascribed to the difference in the shape of peaks between records of the two modes. Line $b$ in Fig. 5 shows the plot obtained by the estimation of peak height with the preparation used in the X-ray study (1). Inconsistency between lines $a$ and $b$ is the result of the reassembly of the cell compartment of the scattering photometer.

DISCUSSION

Molecular weight of the Aspergillus oryzae α-amylase (Taka-amylase A) was determined to be within the range of 50,500 to 51,500 by the combined use of the low angle laser light scattering technique and high pressure silica gel chromatography. No appreciable difference was observed in molecular weight between the two preparations one of which was used in the X-ray study (2).

The molecular weight of the α-amylase was first elaborately estimated by Isemura and Fujita using the sedimentation-diffusion method to be 51,000 (14). As mentioned in the introduction, the molecular weight has been variously reported. The most recent estimate is 50,000 ± 1,000 obtained by the parallel use of the sedimentation equilibrium technique and the molecular sieve technique (15). The above two values are in good agreement with that obtained in the present study.

The present result is reported notwithstanding the following reasons. 1) It was felt that the confirmed molecular weight of the α-amylase estimated by recently developed reliable technique was worth publishing as the previously reported values cited above are somewhat open to doubt, because of the assumption of a non-hydrated ellipsoidal shape for the molecule in calculation (14) and the inappropriate use of a phosphate buffer (for reasons, see "MATERIALS AND METHODS") throughout the measurements (15).

2) The present study was partly carried out as an assessment study of a new technique, the combined use of high pressure silica gel chromatography and the low angle laser light scattering technique.

The combined technique became available in the field of protein characterization only recently as the result of the development of a gel matrix which can be used in aqueous solvents (4, 5). Fukuda et al. (7) first applied the combined technique to proteins, and used a low angle laser light scattering photometer and a differential refractometer as monitors of $M_c$ and $c$, respectively. Molecular weight of a sample protein was estimated using the plot of the ratios, $(Output)_{LS}/(Output)_{RI}$, versus molecular weights of standard proteins as a calibration line. Both sample proteins and standard proteins were presumed to be homologous and "homopolymer-like" by assuming $(dn/dc)$ in Eq. 2 to be constant. The above assumption does not always hold, especially when one is dealing with a protein with non-proteinous components. A slight difference, as much as 4%, in $(dn/dc)$ between bovine serum albumin and the other two proteins examined in the present study may be attributable to the presence of carbohydrate (ca. 3% (w/w)) in the latter two (16). For accurate estimation of protein molecular weights, we believe that the present method is preferable to that of Fukuda et al. (7).

Although the description of details of technique is out of the scope of the present paper, the following points should be noted. 1) Dust particles can be effectively eliminated by filters. Noise and base-line drifts which may occur during the recording of scattering intensity, therefore, mainly come from air-bubbles. Their intrusion into and formation in the flow-line must be carefully avoided. 2) Increase of bacteria both in the reservoir of buffer solution and in the flow-line causes plugging of filters and a noisy record of scattering intensity. Addition of a germicidal agent such as sodium azide to the buffer used is quite effective to prevent this. 3) Both detectors are sensitive to pressure, and a series of measurements, therefore, must be carried out under strictly controlled conditions. A constant flow rate must be kept during the measurements. It is recommended that proteins used as calibration standards have molecular weights near that of the sample protein, as in the present study, and are measured in succession with the latter.

The combined use of high pressure silica gel chromatography and the low angle laser light scattering technique is an effective approach to the determination of molecular weights of large proteins.
scattering technique is quite promising as simple and reliable way to estimate molecular weights of proteins. It is much simpler than the conventional light scattering technique and free from the ambiguity associated with methods depending on the molecular sieving effect such as sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

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