Fluorospectral Intensity of High-molecular-weight Dextrans: Application to the Enzyme Reactions

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A rapid and simple assay procedure of high-molecular-weight polysaccharides is required for the activity measurement of polysaccharide-synthesizing or -hydrolyzing enzymes. When an aqueous solution of dextran was assayed fluorospectrometrically at a fixed wavelength of 340 nm for both excitation and emission, a strong response based on Rayleigh's light scattering was observed. There was a linear correlation between the spectral intensity and concentration of the native dextran, and the intensity was proportional to the molecular weight of the dextran. Two model experiments verified the applicability of fluorospectrometric analysis. That is, a kinetic measurement of dextran synthesis by this method corresponded well with the results evaluated by the reducing end group analysis. Differences between endo-type and exo-type dextranases were clearly demonstrated. Moreover, the endodextranase action on the dextran could be followed by the changes in spectral intensity of the incubation mixture placed in the cuvette. Although fluorospectrometric analysis is applicable to polysaccharides other than dextran, its use is restricted to homogeneous substrates or enzymes. High-performance liquid chromatography with a gel-filtration column indicated that the spectral intensity was specific for the high-molecular-weight dextran, while a differential refractometer gave responses to all sugars including those with low-molecular-weights.

Enzymic reactions of the synthesis or degradation of a polysaccharide are quantified by measurements of the concentration and/or size of the reaction products. There are several physicochemical techniques, such as gel filtration, viscometer, ultracentrifugation, and osmometer,¹⁻³⁾ available for the estimation of the molecular weights of poly- or oligosaccharides. The light scattering method is well established for this purpose.¹⁾ However, more convenient and simple procedures may be useful for the measurement of polysaccharides in enzyme reactions.

Because of progress in high-performance liquid chromatography (HPLC), fluorospectrometers have become widely used because of their high sensitivity to fluorescent derivatives of various compounds. In this paper, we describe the use of the fluorospectrometric method to measure the concentration of a high-molecular-weight dextran, and some

practical applications of this method for enzymic studies.

MATERIALS AND METHODS

Materials. Water-soluble, native dextrans from *Leuconostoc mesenteroides* NRRL B-512F, B-1299, B-1307, B-1355, and B-1416 strains were prepared as described previously.⁴⁾ Dextranase from *L. mesenteroides* NRRL B-512F was prepared as described.⁵⁾ Standard dextrans, T-10 to T-500, were the products of Pharmacia Fine Chemicals. Oyster glycogen (G-8751) and α -amylase from *Bacillus subtilis* (A-6380) were the products of Sigma Chemical Co., St. Louis, MO. Soluble starch (potato), endodextranase from *Chaetomium gracile*, and crystalline glucoamylase from *Rhizopus niveus* were purchased from Seikagaku Kogyo Co., Japan. Mannan from *Saccharomyces cerevisiae* was provided by Dr. T. Nakajima in our laboratory. Other reagents were of the analytical grade.

Assay of dextranase. The initial velocity in the dextranase reaction was measured by incubating 50 μ l of a diluted enzyme solution (0.1 units/ml) with 100 μ l of

sucrose solution in 40 mM acetate buffer (pH 5.2) and 100 μ l of the 0.2% solution of native dextran from *L. mesenteroides* B-512F for 20 min at 30°C. The reaction was stopped by the addition of 250 μ l of NaOH (final 0.2 M), followed by dilution with 2.5 ml of water. Then, the reaction mixture was assayed for reducing sugars (the Nelson-Somogyi method)⁶⁾ and by fluorospectrometry. Relative intensities were measured by a JASCO fluorospectrometer FP-550 (Nippon Bunko Co., Japan). Wavelengths of excitation and emission were fixed at 340 nm.

Assay of dextranases. In advance, the relative activities of endodextranase and glucoamylase, as an exodextranase,⁷⁾ were adjusted to about 0.09 units/ml enzyme solution. The reaction mixtures contained 50 μ l of dextranase and 400 μ l of B-512F native dextran (26.4 mg/ml) in 40 mM acetate buffer (pH 5.2). After incubation at 30°C for suitable periods, the reaction was stopped by the addition of 50 μ l of 1 M NaOH. Samples were analyzed by the Nelson-Somogyi method and by fluorospectrometry.

Assay of amylases. The activities of α -amylase and glucoamylase were adjusted to produce an equivalent amount of reducing sugar per min (about 0.075 units/ml enzyme solution). The reaction mixture contained 150 μ l of enzyme and 300 μ l oyster glycogen (2.5 mg/ml) in 50 mM phosphate buffer (pH 6.0). After inactivation by NaOH, the fluorospectral intensity of the each digest was measured as described above.

HPLC analysis. High-performance liquid chromatography (HPLC) was done with a Waters M45J chromatograph with a gel filtration column of TSK G4000 SW (Toyo Soda Co.). Samples were eluted from the column with water and peaks were detected either with a differential refractometer (Waters, R401), UV detector (JASCO, UVIDECE 100-IV recorder, Nippon Bunko Co.), or a fluorospectrometer (JASCO FP-550).

RESULTS AND DISCUSSION

Detection of dextran by fluorospectrometry

During the course of experiments in which we tried to introduce some fluorescent reagents such as dansyl hydrazine⁸⁾ or 2-aminopyridine⁹⁾ into polysaccharides, we found that an aqueous solution of polysaccharide with high molecular weight gave a strong response on the fluorospectrometer. When the excitation (emission) wavelength was fixed at 340 nm, for example, a water-soluble, native dextran of *Leuconostoc mesenteroides* gave two peaks at 340 and 680 nm of emission (ex-

FIG. 1. Scanning Pattern of Dextran Solution by a Fluorospectrometer.

Native dextran solution (2 mg/ml water) of *Leuconostoc mesenteroides* NRRL B-1416 was placed in the quartz cuvette of 10 mm light pass (3.5 ml quantity). (a) The emission wavelength was fixed at 340 nm and the excitation spectrum was measured. (b) The excitation wavelength was fixed at 340 nm and the emission spectrum was measured. Both sensitivity and selector of JASCO FP-550 fluorospectrometer were adjusted to 1/10 range.

citation) wavelengths (Fig. 1). Although these peaks are attributable to Rayleigh's light scattering,¹⁰⁾ use of a fluorospectrometer with the fixed wavelengths of 340 nm for both excitation and emission enabled us to find the quantitative relationship between the concentration of polysaccharides and their spectral intensity.

Fluorospectral intensities of various saccharides are summarized in Table I. An opalescent polysaccharide solution such as dextran or glycogen, was inclined to give a higher intensity. The difference of intensities among the native dextrans of five strains of *Leuconostoc* may be mainly ascribed to the differences in molecular weight and structure, i.e., types and contents of non- α -1,6 linkages of dextran.

Aqueous solutions of mono- and disaccharides gave no appreciable spectral intensity. Buffer solutions of 40 mM acetate (pH 5.2) and phosphate (pH 6.0) gave the same results (Table I). Therefore, the fluorospec-

TABLE I. FLUOSPECTRAL INTENSITY OF VARIOUS SACCHARIDES AND SOME OTHER COMPOUNDS^a

Compound	Relative intensity	Compound	Relative intensity
Native dextran	B-512F 972	Oyster glycogen	283
	B-1299 887	Soluble starch	112
	B-1307 795	Yeast mannan	63
	B-1355 1130	D-Fructose	15
	B-1416 530	D-Glucose	3
Standard dextran	T-70 7	Sucrose	0
	T-110 9	40 mM acetate buffer (pH 5.2)	3
	T-250 14	50 mM phosphate buffer (pH 6.0)	5
	T-500 26	Bovine serum albumin	22

^a Concentration of the saccharides used was as follows: Native dextrans, standard dextrans, oyster glycogen, soluble starch, and yeast mannan were 1.25 mg/ml water; D-fructose and D-glucose, 10 mg/ml; Sucrose, 100 mg/ml; bovine serum albumin, 2.5 mg/ml. The molecular weights of polysaccharides were as follows: five native dextrans, $2 \times 10^7 <$ (eluted at the void volume of a Sepharose CL-2B column); glycogen, 3.5×10^5 ; soluble starch, $2 \times 10^7 <$; and mannan, 1.3×10^5 . These values were calibrated by standard dextrans T-10 ~ 500, which represented the weight-average molecular weight measured by light scattering.

FIG. 2. Relationship between the Fluorospectral Intensity and the Concentrations of Various Polysaccharides.

(a) Water-soluble, native dextrans from *L. mesenteroides*. ●, B-512F dextran; ○, B-1416 dextran; ▼, B-1307 dextran; ▽, B-1355 dextran. (b) Glucans and mannan. ○, soluble starch; ●, oyster glycogen; ▼, yeast mannan. (c) B-1416 Dextran ($13 \sim 328 \mu$ g/ml) was measured with a 10-fold increase, from the range of 1/10 to 1, of the selector.

trometric measurement was not affected by low-molecular-weight sugars or inorganic buffer solutions. Bovine serum albumin (2.5 mg/ml) gave a higher response than these sugars.

Response to the dextran concentration and molecular weight

As shown in Fig. 2a, four water-soluble, high-molecular-weight dextrans from *L. me-*

senderoides gave linear calibration curves up to 2.5 mg/ml concentration. Moreover, oyster glycogen, soluble starch, and yeast mannan gave linear correlations (Fig. 2b). These values were obtained at a normal sensitivity of the fluorospectrometer and a 10-fold increase in the sensitivity showed a linear correlation of spectral intensity and a much lower concentration of dextran ($13 \sim 330 \mu$ g/ml) as shown in Fig. 2c. Thus, the detection limit is comparable.

with that of the well-known colorimetric phenol-sulfuric acid method.¹¹⁾ Although the relative intensity of standard dextrans (T-series) was small, there was a linear correlation between the molecular weight of standard dextrans and their spectral intensity (Fig. 3). Since the molecular weights of these dextrans were measured by the light scattering procedure,¹²⁾ the use of a fluorospectrometric assay instead of a light scattering apparatus would give satisfactory results.

Application to the dextranucrase reaction

Dextranucrase (sucrose: 1,6- α -D-glucan 6- α -D-glucosyltransferase, EC 2.4.1.5) from *L. mesenteroides* is known to produce a high-molecular-weight α -glucan (dextran) from sucrose.¹³⁾ The activity of this enzyme could be measured by either the released reducing sugar or the synthesized dextran.¹⁴⁾ Because of the very small spectral intensities of low-molecular-weight sugars such as sucrose and D-fructose (the substrate and reaction product of dextranucrase, respectively) shown in Table I, the accuracy of measurement of enzymically synthesized dextran in the reaction mixture could not be affected by these sugars. Therefore, kinetic experiments were done to measure the amount of synthesized dextran

specifically. As shown in Fig. 4a, the dextranucrase activity was strongly inhibited by EDTA, where the reducing sugar was measured by the Nelson-Somogyi method. Similarly to our previous results,¹⁵⁾ the reciprocal plot gave biphasic lines indicating an allosteric property of dextranucrase, and two K_i values (0.1 mM and 1.1 mM) were estimated for the limbs of the line. Fluorospectrometric assay of the synthesized dextran gave almost the same results as that of the reducing sugar

FIG. 3. Relation between the Fluorespectral Intensity and the Molecular Weight of Various Standard Dextrans.

The weight-average molecular weight of standard dextrans (5 mg/ml) used were T-10, (M_r 10,000); T-20, (20,000); T-40, (40,000); T-70, (70,000); T-110, (110,000); T-250, (250,000); T-500, (500,000).

FIG. 4. Inhibition of the Dextranucrase Reaction by EDTA.

(a) Dixon plot of the enzyme activity assayed for the released reducing sugars by the Nelson-Somogyi method. (b) Dixon plot of activity assayed for the synthesized dextran by the fluorospectrometer. Incubation was done in the presence of 146 mM (●) and 46 mM (○) of the substrate, sucrose. Scales of y-axis represented as $1/v$ (μmol reducing sugar as D-fructose/min/ μg enzyme) $^{-1}$ for (a) and $1/v$ (μg dextran/min/ μg enzyme) $^{-1}$ for (b).

analysis (Fig. 4b). The K_i values were 0.1 mM and 2.4 mM. In both cases, EDTA showed noncompetitive and competitive types of inhibition for the substrate sucrose. These results clearly proved that a simple fluorospectrometric assay of the synthesized dextran was useful for kinetic measurements.

Application to the dextranase reactions

The action of endo-type polysaccharide hydrolases rapidly decreases the molecular weight of the substrate, which is generally measured by the decrease in viscosity.¹⁶⁾ In contrast, the action of exo-type hydrolases decreases the molecular weight of the substrate more slowly. Therefore, the fluorospectrometric method was used to distinguish the action patterns of endo- and exo-type of enzymes. Changes in the relative spectral intensity were measured during the course of

the enzymic degradation of dextran with a typical endodextranase from *Chaetomium gracile* and with a glucoamylase from *Rhizopus niveus* (Fig. 5). The latter enzyme is known to release D-glucose from the nonreducing ends of dextran.⁷⁾ When the amounts of two enzymes were adjusted to produce an equivalent amount of reducing sugar per min, there was a great difference between the action of endodextranase and glucoamylase. The endodextranase gave a prompt decay curve of spectral intensity representing a decrease in high-molecular-weight dextran, but the glucoamylase gave only a small decrease throughout the reaction.

Similar comparisons were made with amylases and the oyster glycogen system. Again the endo-type α -amylase gave a rapid decrease in the spectral intensity compared with the exo-type glucoamylase, as shown in Fig. 5b.

FIG. 5. Action of Endo-type and Exo-type Glucanases on the High-molecular-weight Substrates.

(a) Endodextranase from *C. gracile* (●, ▼) and glucoamylase, as an exodextranase from *R. niveus* (○, ▽) were mixed with the native dextran of *L. mesenteroides* B-512F. Relative fluorospectral intensity (●, ○) and reducing sugar (▼, ▽) were measured at the periods indicated. (b) α -Amylase from *B. subtilis* (●) and glucoamylase from *R. niveus* (○) were mixed with the oyster glycogen, and their relative fluorospectral intensity was measured. (c) Endo-dextranase was mixed with the native B-512F dextran (2%) in a 3.5 ml quartz cuvette, and the change in fluorospectral intensity was recorded. Reaction was done at 24°C. Lines I, II, and III corresponded to the enzyme of 0.9, 0.3, and 0.09 units/ml, respectively. For details see MATERIALS AND METHODS.

FIG. 6. HPLC Analysis of the Endodextranase Digested Dextran with a TSK G4000 SW column.

Samples were prepared as described in the MATERIALS AND METHODS and Fig. 5a. Sample numbers (#1~6) indicated in the charts corresponded to 0, 2, 4, 8, 12, and 16 min hydrolyzates. Detectors used were (a) differential refractometer, (b) fluorospectrometer, and (c) UV detector at 220 nm. Each 20 μ l sample was injected to the column.

Moreover, changes in the spectral intensity could be directly monitored by a reaction conducted in the cuvette. The endodextranase was mixed with the substrate dextran and decreases in spectral intensity were recorded on a chart (Fig. 5c). Together with the results shown in Fig. 5a, the fluorospectrometric method was shown to be sensitive enough to detect the change in molecular weight of the substrate in a short time. The latter reaction system might be effective for the kinetic analysis of endodextranases and α -amylases with a rapid assay system such as the stopped-flow method.

Application to the HPLC system

For our previous paper, we measured dextran by a UV spectrometer.¹⁷⁾ Like the current fluorospectrometric method, the UV method is specific to high-molecular-weight polysaccharides. Thus, to characterize the reaction products, the above two methods were compared referring to the differential refractometer, which responds to carbohydrate of both low and high molecular weights.

Samples of the endodextranase-digested dextrans shown in Fig. 5a were subjected to

HPLC and separated with a TSK G4000 SW gel filtration column. The elution patterns were monitored by a differential refractometer, UV detector, or fluorospectrometer. As shown in Fig. 6a, the response on a differential refractometer revealed time-dependent changes in the molecular weight of the product and almost no peak was shown at the void volume after 16 min of digestion. There was a very broad peak at the low-molecular-weight regions. In contrast, UV and fluorospectrometric response gave a major peak at the void volume, which decreased as the hydrolysis of dextran with the endodextranase proceeded, and the low-molecular-weight products gave no response. In the case of differential refractometer and UV, the endodextranase gave a protein peak at the elution time of 14.8 min (Fig. 6a and c).

The advantage of the fluorospectrometric method is that the amount of high-molecular-weight polysaccharides in the reaction mixture could be measured directly without any pretreatment or reagents. Because linear correlations were found between the spectral intensity and polysaccharide concentrations or molecular weight, the changes in molecular

weight caused by the endo-type of enzymes could be sufficiently evaluated by our procedure (Fig. 5). In conclusion, our results provide a convenient assay procedure for high-molecular-weight polysaccharides in reaction mixtures containing either synthesizing or degrading enzymes of polysaccharides.

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