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Preparation and Solution Properties of Pullulan Fractions as Standard Samples for Water-soluble Polymers

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SUMMARY

A series of pullulan fractions with molecular weights in the range 5×10^3 to 8×10^5 were prepared. The weight-average molecular weight (M_w) of all the samples was determined by sedimentation equilibrium. The hydrodynamic properties of pullulan in aqueous solution were investigated by viscometry and ultracentrifugation. The experimental results indicate that pullulan molecules in water are fairly stable and behave as expanded random coils when M_w is above 2×10^4. The molecular weight distributions of the fractions were measured by gel filtration. The ratio M_w/M_n was close to 1·1, except for a sample with the highest M_w.

It is concluded that the pullulan fractions prepared by the present work are well characterized and have a narrow molecular weight distribution. They may be useful as standard samples for studies of water-soluble polymers.

INTRODUCTION

The availability of a series of well-characterized polymers which can be used as reference samples is essential for the calibration of chromato-

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graphic methods and also for various other experiments concerned with the molecular weights of polymers. Requirements for standard polymer samples are as follows: (i) the average molecular weight should be known; (ii) the molecular weight distribution of each sample should be narrow; (iii) a series of samples with a wide range of molecular weight should be available; (iv) the samples and the solution should be stable and be handled without special precautions; (v) the molecular structure and the conformation in solution are simple and well-characterized, and may be described using established theories.

A few polymers satisfying the above requirements are commercially available, e.g. polystyrene. These are, however, not water soluble. Dextran is frequently used as a calibration standard for aqueous systems. Although it is possible to prepare dextran fractions with a very narrow molecular weight distribution (e.g. Basedow & Ebert, 1979), dextran samples that are usually prepared or are readily available have a rather broad distribution. The degree of branching in the dextran molecule depends on the sample species and on the molecular weight, and hence the molecular weight dependence of the solution properties such as the relation between the molecular weight and the intrinsic viscosity (Granath, 1958) may differ from sample to sample. Therefore, other linear polymers which are more suitable as standards than dextran are desirable.

Pullulan, a polysaccharide produced from a cultivated fungus of *Aureobasidium pullulans (Pullularia pullulans)*, is an α-glucan mainly consisting of maltotriose as repeating units linearly jointed through α-1,6 glycosidic linkages (Ueda *et al.*, 1963; Wallenfels *et al.*, 1965; Catley & Whelan, 1971), as shown in Fig. 1. From hydrolysis studies with acid and the enzyme pullulanase (EC 3.2.1.41) it has been concluded that there is no branching in the chain structure (Taguchi *et al.*, 1973).

Pullulan is an amorphous, edible and naturally degradable polymer, which is readily water soluble. Industrial production has been developed in Japan (Hayashibara Biochemical Laboratories, Inc.) from fermentation of partially hydrolysed starch and special samples of pullulan can also be produced from sucrose.

Until recently, no reliable studies on the molecular characteristics and solution properties of pullulan have been reported. We have carried out preliminary experiments on unfractionated pullulan samples (Ohta

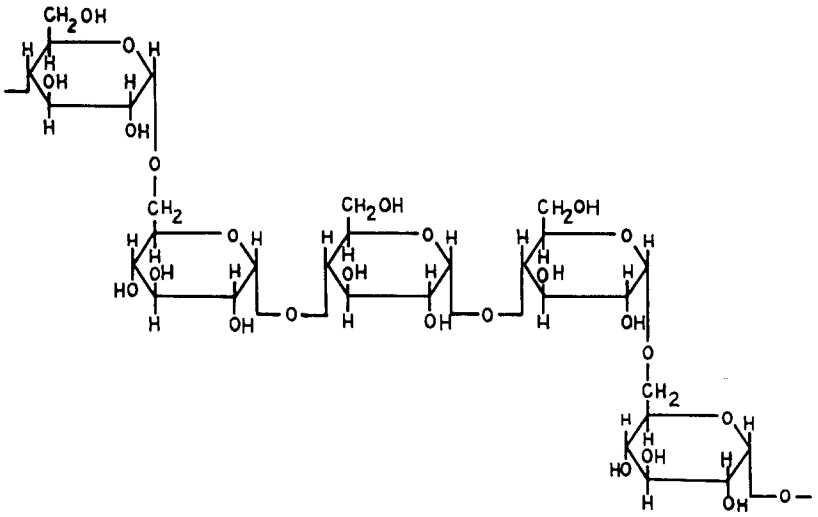

Fig. 1. Chemical structure of pullulan.

et al., 1979) which indicated that pullulan may behave as a typical flexible linear polymer. It is stable in aqueous solution and shows no anomalous behaviour such as crystallization and aggregation.

Calculation on the configuration statistics of α-glucan in solution showed that polymer chains containing α-1,6 linkages have substantial conformational freedom (Brant & Burton, 1981). Recently Kato *et al.* (1982) investigated experimentally the solution properties of pullulan in water and confirmed that the pullulan chain has considerable flexibility, behaving as an expanded random coil in aqueous solution.

These properties suggest that pullulan may be useful as a standard sample for water-soluble polymers. We have therefore prepared a series of pullulan fractions, both for our own use and for the benefit of other workers.

During the preparation of this paper, Kato *et al.* (1983) reported that pullulan is useful as a polymer standard in aqueous gel permeation chromatography and a series of pullulan samples have become commercially available.

We describe in detail the preparation of considerable quantities of pullulan fractions. The characterization of these fractions by sedimentation, viscosity and gel filtration is also reported.

EXPERIMENTAL

Materials

The original samples of pullulan used were products of Hayshibara Biochemical Laboratories Inc., Japan. Some of the samples were produced especially for this work. For example, the pullulan sample, from which the high molecular weight fraction was prepared, was produced from sucrose, and samples for fractions with molecular weights lower than 5×10^4 were prepared from a commercial product by partial degradation with pullulanase. The sample of the lowest molecular weight was made by further degradation in 0·1 M HCl aqueous solution at 80°C. The molecular weights of these products were adjusted approximately to the desired values by controlling the degree of fermentation or the degradation reactions.

Organic solvents were purified by distillation. Water, used as solvent for measurements of the solution properties, was re-distilled in a Pyrex glass apparatus. Other chemicals were used without further purification.

Preparation of standard samples

The preparation of each pullulan fraction consisted of three steps: (i) removal of impurities; (ii) coarse fractionation; and (iii) fine fractionation.

Because it was desired to prepare large quantities of pullulan fractions (> 100 g), if reprecipitation were performed by pouring dilute solution of a whole crude sample into the precipitant, a very large amount, e.g. 1000 liters, of solvent would be needed. Therefore, the following procedure was adopted to remove impurities.

About 1·5–2·5 kg crude pullulan was dissolved in 50–60 liters water, and methyl alcohol added to the solution with stirring until a gel was formed. The gel was transferred into less than 100 liters of an appropriate mixture of water and methyl alcohol and stirred for several hours to extract impurities. About 80% of the crude sample was retained in the gel which was then diluted with water and subjected to coarse fractionation. This was carried out in a 300 liter vessel (thermostated at 30°C) by successive precipitation with methyl alcohol. The initial concentration of pullulan in the solution was *c.* 2×10^{-2}–4×10^{-2} g cm^{-3}, depending on the molecular weight. An example of the fractiona-

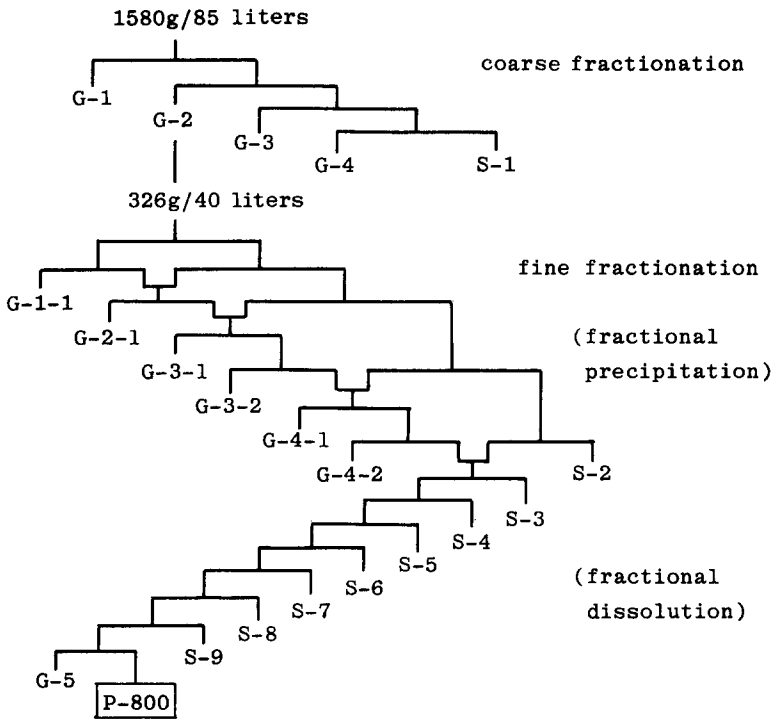

Fig. 2. Schematic diagram of large scale fractionation for preparation of standard sample P-800. A part of stock sample G-2, prepared by coarse fractionation (see text), was subjected to the fine fractionation procedure. The gel separated by the first fractionation step was diluted with water and refractionated to remove a small amount of the gel phase G-1-1. The supernatant phase was combined with the gel phase separated by the second fractionation step and then refractionated. A similar procedure was repeated. After the separation of supernatant S-2, a mixture of water and methyl alcohol was added to the gel phase to dissolve a small amount of the low molecular weight fraction. This procedure of fractional dissolution was repeated to remove fractions S-3, S-4, etc. Finally, a small amount of gel G-5 was separated and the main fraction P-800 was obtained from the supernatant.

tion scheme is shown in Fig. 2. Appropriate fractions, called stock samples, were selected for fine fractionation.

Methods and conditions for the fine fractionation were first investigated using small amounts of the stock samples. After several trials, it was found that refractionation by conventional procedures was not very effective with pullulan fractions, and we decided to prepare only

one fraction with a narrow molecular weight distribution from each of the stock samples using repeated precipitation- and dissolution-fractionations in dilute solution.

The fine fractionation was performed with the same solvents as those for the coarse fractionation, but the initial concentration of the solution was reduced to *c.* 1×10^{-2} g cm^{-3}. As shown in Fig. 2 and described in the caption, refractionation was repeated to remove high molecular weight fractions G-1-1, G-1-2, etc. Then, low molecular weight portions were separated repeatedly by fractional dissolution. Care was taken to ensure equilibrium was reached for all of the fractionation procedures.

The final fraction of this multiple fractionation was purified by precipitation in methyl alcohol and dried *in vacuo* for 6 h at 90°C. The amounts of the standard samples obtained were one-third or less of the amounts of the initial stock samples subjected to the fine fractionation.

Preparation of semi-standard samples

As will be mentioned later, the molecular weight distributions of the standard samples were determined by gel filtration. This method relies on calibration with well-characterized samples with narrow molecular weight distributions. The calibration can be performed using the standard samples themselves within their molecular weight range, but requires additional fractions having molecular weights lower and higher than this range. Two pullulan fractions, called semi-standard samples, were prepared by fractionation using gel columns.

Figure 3 shows the scheme for the preparation of a very low molecular weight fraction P-3 (see figure caption). Another fraction of high molecular weight was prepared from the stock sample with a molecular weight of *c.* 140×10^4 by a rather similar procedure to that used for the low molecular weight material. In this case a Sepharose CL-2B (5×100 cm) column was used. Because of the small amounts of materials that could be applied to the column, the first fractionation (elution of 75 cm^3 of 0·2% (w/v) solution) was repeated 22 times and refractionation (elution of 80 cm^3 of 0·1% solution) was repeated nine times. 0·35 g sample P-1200 was obtained from 3 g of the stock sample. This sample was dried in a desiccator at room temperature, because high molecular weight material may be slightly degraded at high temperatures.

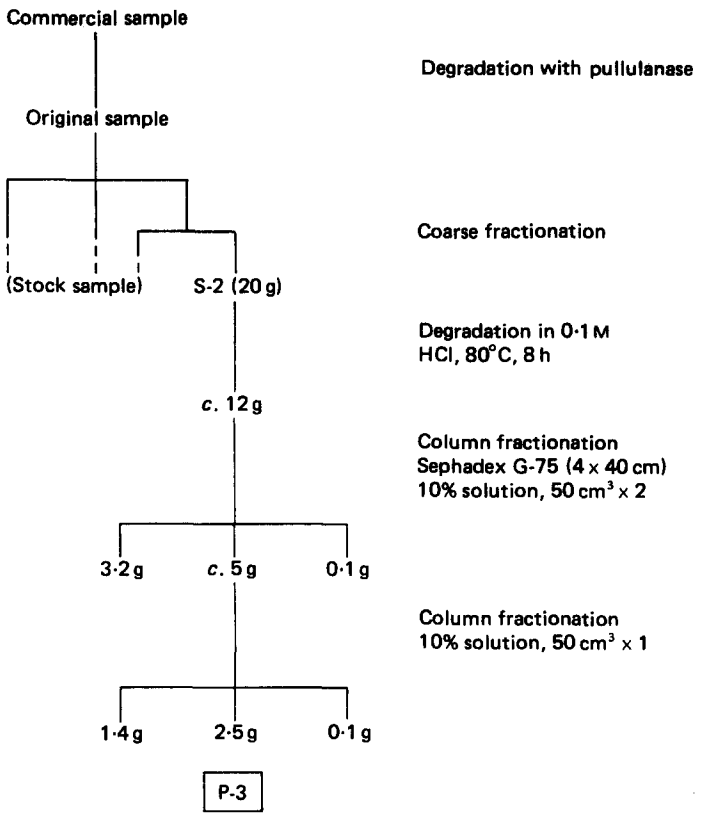

Fig. 3. Schematic diagram of the preparation for semi-standard sample P-3. Very low molecular weight pullulan was prepared by degradation with HCl from one of the stock samples. 10 g of this sample was dissolved in 100 cm^3 of 0·9% NaCl aqueous solvent contained 0·02% NaN_3. Each 50 cm^3 of the solution was chromatographed on a jacketed Sephadex G-75 gel column (4·0 x 40 cm). The middle fraction was concentrated by a rotary evaporator, then refractionated into three fractions using the same column.

Ultracentrifugation

Sedimentation experiments were performed in a Beckman Spinco Model E ultracentrifuge equipped with an electronic speed control system. Molecular weights were determined in water at 25°C by the conventional sedimentation equilibrium method (Chervenka, 1970).

A filled-Epon 3·0 cm cell was used for most of the measurements and a 1·2 cm cell was used for solutions of high concentration. The length of the solution column in a cell was 0·22–0·30 cm, depending on the experimental conditions. Since a very long period would be required to attain sedimentation equilibrium for the highest molecular weight sample in such a long solution column, P-1200 was measured in 0·02% NaN_3 with a column length less than 0·20 cm, in which the period of sedimentation was about three days. Rotor speed was selected to make the product of M_{app} and λ (see equations below) close to unity for each run. The photographed Rayleigh fringe patterns were read using a Nikon Shadowgraph model 6.

The weight-average molecular weight M_w was evaluated according to the following equations (Fujita, 1969).

$$M_{app} = (c_b - c_a)/\lambda c_0$$

$$\lambda = (1 - \bar{v}\rho)(r_b^2 - r_a^2)\,\omega^2/2RT$$

$$M_{app}^{-1} = M_w^{-1} + 2A_2(1 + \delta_1)\,\bar{c}$$

$$\bar{c} = (c_a + c_b)/2$$

where r_a and r_b are the radial distances from the center of rotation to the meniscus and to the bottom of the solution column, respectively; c_a and c_b are the polymer concentrations in equilibrium at r_a and r_b, respectively; c_0 is the initial concentration; $\bar{v}$ is the partial specific volume of the polymer; ρ is the density of the solvent; ω is the angular velocity; R is the gas constant; T is the temperature; A_2 is the second virial coefficient; δ_1 is the correction term for polydispersity. Observed values of M_{app}^{-1} were plotted against $\bar{c}$ and extrapolated to infinite dilution. The correction term δ_1 for the samples with narrow molecular weight distribution may be less than the experimental error and was neglected in the evaluation of A_2. The M_w values of the unfractionated samples were approximated by the M_{app} observed in a solution of concentration 5×10^{-4} g cm^{-3}. The relation between the fringe displacement and the concentration of the polymer was determined for each sample by using a capillary type synthetic boundary centrepiece. The value of $\bar{v}$ in water at 25°C was determined by measurements of the solution densities using a 30 cm^3 pycnometer.

The values of M_z/M_w and the z-average molecular weight M_z were evaluated according to the method of Norisuye *et al.* (1980). In this

analysis, apparent values of $(M_z/M_w)^{-1}$ were calculated from the values of $d(\ln c)/dr$ at r_a and at r_b, then extrapolated to infinite dilution. The values of $d(\ln c)/dr$ were estimated graphically from the plot of $\ln c$ versus r^2, and may lead to considerable error in the values of M_z/M_w and M_z.

Sedimentation coefficients (s) in water at 25°C were determined for the standard samples with the exception of P-10 and P-5. Schlieren optics and a 1·2 cm aluminum single sector cell were generally used, though a filled-Epon synthetic boundary cell was employed for a few solutions of P-20. The value of s_0 at infinite dilution and the coefficient of concentration dependence k_s were evaluated from the plot of s^{-1} against c according to the equation:

$$s^{-1} = s_0^{-1}(1 + k_s c)$$

Viscosity

Solution viscosities in water at 25°C were measured with Ubbelohde-type capillary viscometers. No kinetic energy correction was made. Intrinsic viscosities $[\eta]$ were evaluated from both plots of η_{sp}/c versus c and $(\ln \eta_r)/c$ versus c, where η_{sp} and η_r are, respectively, the specific viscosity and the relative viscosity of the solution.

To investigate the stability of high molecular weight pullulan in solution, P-1200 was dissolved in pure water and in water containing 0·02% NaN_3 and stored at 25°C. The intrinsic viscosity was measured several times over a 10-day period.

The concentrations of most of the solutions used for the measurement of viscosity and other solution properties were obtained from the dry weight determined by drying a portion of the solution *in vacuo* for 4 h at 110°C.

Gel filtration

A number of preliminary experiments were performed to investigate the use of gel filtration in determining the molecular weight distribution of small quantities of pullulan. Various kinds of gel applicable to normal pressure columns were tested; these included columns packed with one kind of gel in the lower part and layered with another gel in the upper part of the column. It was concluded that any one column

could not measure the whole molecular weight range covered by the pullulan samples. Therefore, depending on the molecular weight of the sample, one of three columns packed with Sepharose CL-2B, Sepharose CL-6B, or Sephadex G-75 were used.

Columns were 1·5 cm in diameter and 100 cm in length with a jacket circulating water at a constant temperature of 20°C. The solvent used was 0·9% aqueous NaCl solution containing 0·02% NaN_3. After equilibration of the gel with the solvent, 1 cm^3 0·5% pullulan solution was applied to the column and eluted with the solvent at a constant flow rate of 6 cm^3 h^{-1} (Sepharose CL-2B) or 12 cm^3 h^{-1} (Sepharose CL-6B and Sephadex G-75) using a peristaltic pump. For the high molecular weight samples (P-1200 and P-800) having high intrinsic viscosities, concentrations of the solutions were reduced to 0·1% and 0·2%, respectively. The concentration of polymer in the eluent was measured by a flow cell type differential refractometer of Shodex RI or Knauer type 61.00 with a recorder.

Void volume of the column was determined before each measurement by using a fraction of blue dextran, which was prepared in our laboratory by fractionation from a commercial product of Pharmacia. The total volume of the column was calculated from the column dimensions and the partition coefficient K_{av} was evaluated from the elution pattern peak.

RESULTS AND DISCUSSION

Stability and electrical charge in solution

Intrinsic viscosities of pullulan samples measured in 0·1 M NaCl are in good agreement with results obtained in pure water (Ohta *et al.*, 1979) and, with the exception of P-1200 (discussed below), no anomalous sedimentation behaviour was observed in water, indicating that pullulan molecules have no electrical charge. Because of this the molecular weight and other solution properties were determined from measurements in water without salts.

There was some evidence that the highest molecular weight pullulan sample degraded in aqueous solution. Thus for P-1200 the intrinsic viscosity in the absence of NaN_3 decreased by about 5% after 10 days' storage at 25°C. The change in viscosity on storage for this length

of time in 0·02% NaN_3 or at 5°C in pure water for one month was negligible. A slight decrease in molecular weight was detected during the three days required for sedimentation equilibrium of P-1200. For this reason measurements were performed in NaN_3. No change in molecular weight was detected during sedimentation of P-800. The reason for the slight degradation of very high molecular weight pullulan at room temperature is not clear. The addition of NaN_3 is effective in preventing degradation in solution.

Molecular weights

Examples of plots of $\ln c$ versus r^2 obtained from sedimentation equilibrium are shown in Fig. 4. The downward curvature for the high molecular weight sample indicates significant non-ideal behaviour, whereas the slight upward curvature for P-10, for which the effect of non-ideality is small, is due to heterogeneity in molecular weight.

The value of $\bar{v}$ of pullulan in water at 25°C was determined as 0·602 $cm^3\ g^{-1}$ and used in calculations based on the ultracentrifuge data.

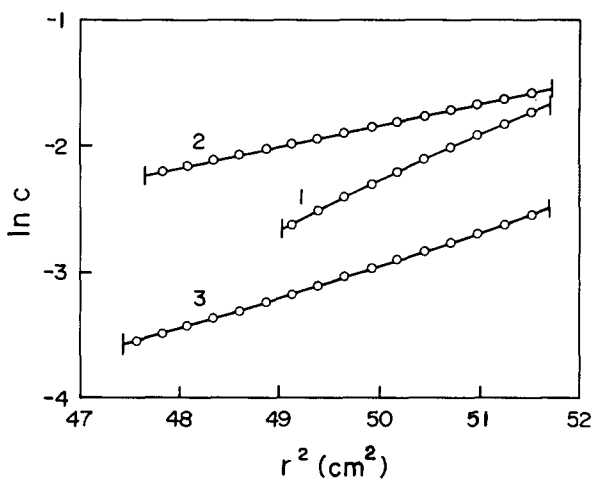

Fig. 4. Typical plots of $\ln c$ versus r^2 from sedimentation equilibrium of pullulan fractions in water at 25°C. Conditions were: 1, P-1200 (contained NaN_3), $c_0 = 0{\cdot}120 \times 10^{-2}$ g cm^{-3}, $(r_b - r_a) = \Delta r = 0{\cdot}190$ cm, 72·5 h at rotor speed of 2400 rpm. 2, P-200, $c_0 = 0{\cdot}153$, $\Delta r = 0{\cdot}291$ cm, 48·3 h at 3400 rpm. 3, P-10, $c_0 = 0{\cdot}049$, $\Delta r = 0{\cdot}304$ cm, 16·2 h at 16 000 rpm.

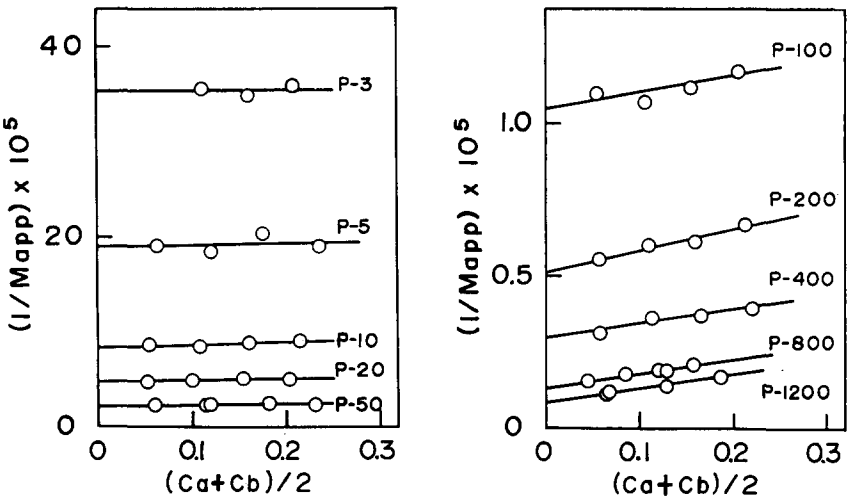

Fig. 5. Plots of M_{app}^{-1} obtained by sedimentation equilibrium against $(c_a + c_b)/2 = \bar{c}$ for pullulan fractions in water at 25°C.

Figure 5 shows the plots of M_{app}^{-1} versus $\bar{c}$ for all samples. The values of M_w and A_2 obtained from these straight lines are summarized in Table 1, together with those of M_z and M_z/M_w. The values of A_2 are slightly larger than those obtained by Kato *et al.* (1982) from light scattering, but the molecular weight dependence of A_2 is close to their results.

The ratio M_z/M_w for most of the samples is smaller than 1·1, indicating a satisfactory narrow molecular weight distribution; however, the values of M_z/M_w for the high molecular weight samples, especially P-1200, may be less reliable due to the significant non-ideality of the solution.

Viscosity and sedimentation

Observed values of $[\eta]$ in water at 25°C for the standard and the semi-standard pullulan samples are summarized in Table 2. A double logarithmic plot of $[\eta]$ versus M_w is shown in Fig. 6, including the data for the unfractionated samples (Ohta *et al.*, 1979). The points for the fractionated pullulan can be fitted by a straight line for molecular weights

TABLE 1
Results of Sedimentation Equilibrium for Standard and Semi-standard Samples of Pullulan in Water at 25°C

Sample	$M_w \times 10^{-4}$	$M_z \times 10^{-4}$	M_z/M_w	$A_2 \times 10^4$ $(cm^3 g^{-1})$
P-1200	123·8	129	1·04	2·2
P-800	75·8	90·2	1·19	2·3
P-400	33·8	39·0	1·15	2·2
P-200	19·4	20·6	1·06	3·5
P-100	9·54	10·9	1·15	2·6
P-50	4·67	5·28	1·13	5·5
P-20	2·08	2·18	1·05	4·9
P-10	1·20	1·30	1·08	13·2
P-5	0·53	0·55	1·04	10·3
P-3	0·28	0·30	1·07	–

TABLE 2
Viscosity and Sedimentation Data for Standard and Semi-standard Samples of Pullulan in Water at 25°C

Sample	$[\eta] \times 10^{-2}$ $(cm^3 g^{-1})$	$s_0 \times 10^{13}$ (s)	$k_s \times 10^{-2}$ $(cm^3 g^{-1})$	$k_s/[\eta]$	$\beta' \times 10^{-6}$
P-1200	2·378	–	–	–	–
P-800	1·766	11·6	2·48	1·40	2·27
P-400	1·026	8·40	1·53	1·49	2·35
P-200	0·709	6·37	0·90	1·27	2·28
P-100	0·440	4·83	0·63	1·43	2·37
P-50	0·282	3·47	0·36	1·28	2·36
P-20	0·163	2·33	0·23	1·41	2·26
P-10	0·116	–	–	–	–
P-5	0·073	–	–	–	–
P-3	0·054	–	–	–	–
			Average:	1·4	2·32

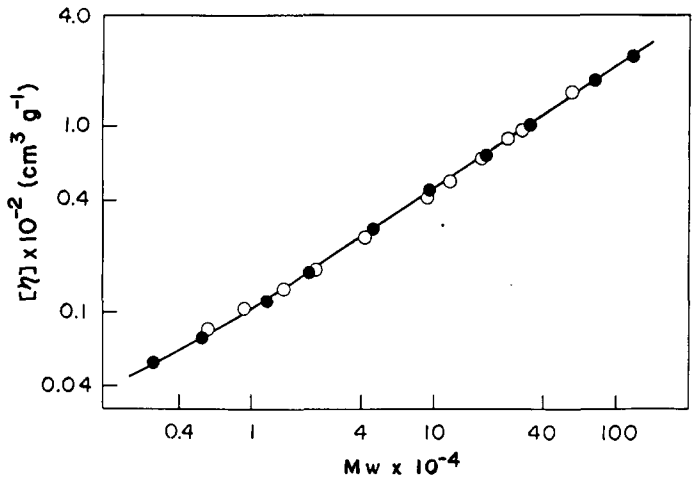

Fig. 6. Double logarithmic plot of $[\eta]$ versus M_w for pullulan fractions (●) and for unfractionated samples (○). The line represents the relation for the fractionated samples.

above 2×10^4. The Mark–Houwink–Sakurada equation obtained is

$$[\eta] = 2{\cdot}36 \times 10^{-2} M_w^{0{\cdot}658} \quad (\mathrm{cm^3\,g^{-1}})$$

This value of the exponent indicates that the pullulan molecule in water behaves as a random coil, swollen by the excluded volume effect, in a good solvent. The data of Fig. 6 are in good agreement with the results of Kato *et al.* (1982), which can be represented by the equation:

$$[\eta] = (1{\cdot}91 \pm 0{\cdot}02) \times 10^{-2} M_w^{0{\cdot}67 \pm 0{\cdot}01}$$

for pullulan fractions with M_w larger than $4{\cdot}8 \times 10^4$.

For molecular weights below 2×10^4, the slope of the double logarithmic plot decreased and was about 0·5 for the lowest molecular weight of 3×10^3. A similar relation was also obtained by Kato *et al.* (1982). It is suggested that the excluded volume effect in water may be reduced or the conformation may be different from a random coil for pullulan with a low molecular weight. Gekko (1971) and Gekko & Noguchi (1971) discussed the conformational change of dextran in water at a molecular weight of around 2×10^3. However, the present experiments

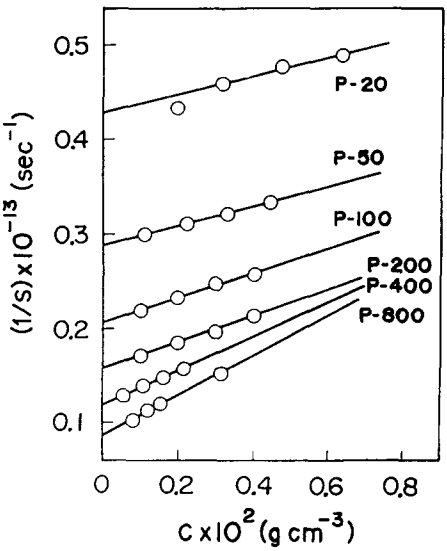

Fig. 7. Concentration dependence of the sedimentation coefficient s for pullulan standard samples in water at 25°C.

are insufficient to support a detailed discussion of conformation, which should be left for further studies on low molecular weight α-glucans.

Figure 7 shows plots of s^{-1} versus c for the standard samples in water at 25°C. The values of s_0 and k_s evaluated from these plots are listed in Table 2. The double logarithmic plot of s_0 against M_w is shown in Fig. 8, together with the relation for dextran fractions in water at 25°C (Ohta *et al.*, 1976). The points for pullulan are fitted by a straight line yielding the following relation for a range of M_w values between 2×10^4 and 80×10^4.

$$s_0 = 2{\cdot}86 \times 10^{-15} M_w^{0.445} \quad \text{(s)}$$

Dextran is a branched polymer and the relation between $[\eta]$ and M (e.g. Granath, 1958) is quite different from that for pullulan. However, the relation between s_0 and M_w for dextran is close to that for pullulan and approximated by a straight line as shown in Fig. 8. It is suggested that the sedimentation coefficient may be much less sensitive than the viscosity to differences in the structure of α-glucans.

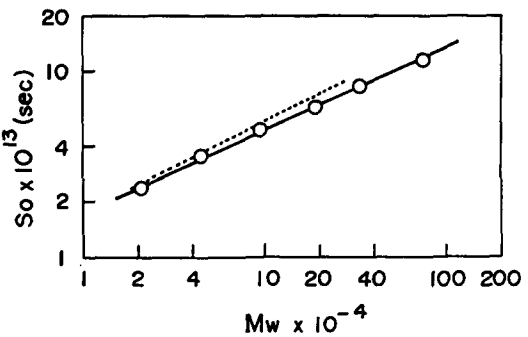

Fig. 8. Molecular weight dependence of s_0 in water at 25°C for pullulan standard samples (——) and for dextran fractions (·····).

The ratio $k_s/[\eta]$ for pullulan is independent of molecular weight (see Table 2) and approximately equal to 1·4, while the ratio for dextran was 2·1 (Ohta *et al.*, 1979).

Hydrodynamic data for polymer solution can be combined by a parameter β' according to the equation derived by Mandelkern and Flory (1952):

$$\frac{N_A s_0 [\eta]^{1/3} \eta_0}{(1-\bar{v}\rho)} = (100)^{1/3} \beta' M^{2/3}$$

where N_A is Avogadro number, η_0 is the solvent viscosity and the factor of $(100)^{1/3}$ arises when $[\eta]$ has units of $cm^3\ g^{-1}$ (Tanford, 1961).

Calculated values of β' from the observed values for M_w, s_0 and $[\eta]$ for the standard samples of pullulan are independent of molecular weight (Table 2). The average value of $2{\cdot}32 \times 10^6$ is reasonable for a random coil polymer in a good solvent (Flory, 1953).

Gel filtration

Figure 9 shows the plots of K_{av} against $\log M_w$ for the standard and the semi-standard pullulan samples. With the exception of the results obtained with Sepharose CL-2B, K_{av} was evaluated from the elution pattern peak. Generally, K_{av} for the peak does not correspond exactly to M_w due to the heterogeneity of the sample. The following method was applied to obtain the calibration curve representing the relation between the elution volume and the molecular weight.

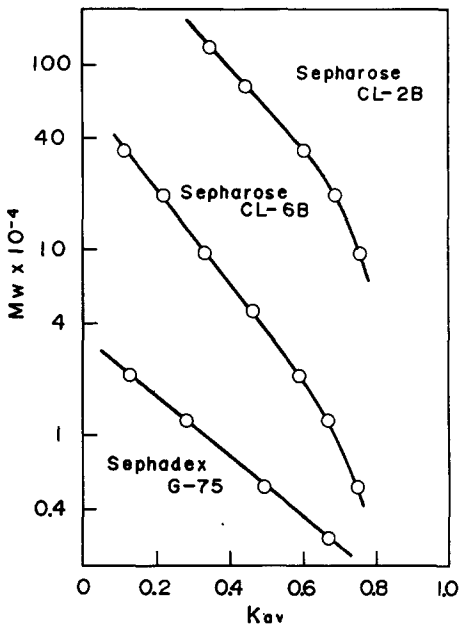

Fig. 9. Relationship between K_{av} and $\log M_w$ of pullulan fractions from gel filtration. K_{av} for Sepharose CL-2B was corrected the effect of heterogeneity of sample (see text).

Using the relationship between K_{av} obtained from the elution pattern peak and M_w, the elution pattern of each sample was converted to an apparent molecular weight distribution. The value of the weight-average molecular weight of this apparent distribution, $(M_w)_{app}$, was calculated and compared with M_w observed by sedimentation equilibrium.

The differences between M_w and $(M_w)_{app}$ evaluated with Sepharose CL-6B and Sephadex G-75 were within experimental error. Therefore, the curves of K_{av} versus M_w without corrections were used for the calibration of these columns. On the other hand, $(M_w)_{app}$ evaluated from results obtained with Sepharose CL-2B column were larger than M_w, indicating that molecular weights were overestimated if the relation between K_{av} and M_w is used directly as a calibration curve. A corrected calibration curve was thus derived where K_{av} was assigned a corresponding value of M_w which was lower than that in the initial calibration by a factor $M_w/(M_w)_{app}$. For Sepharose CL-2B the corrected calibration curve is shown in Fig. 9.

This procedure is only an approximate method for the correction of calibration curves. Nilsson & Nilsson (1974) have reported and discussed a more rigorous method. However, for the well-fractionated samples used in this work where $(M_w)_{app}$ was in good agreement with M_w, the calibration curves shown in Fig. 9 could be employed for the evaluation of their molecular weight distributions.

Molecular weight distribution

Elution patterns for the standard samples of pullulan, for which K_{av} for the peak was in the middle of the calibration curves, were close to a symmetrical Gaussian curve and were converted to molecular weight distribution curves using the relationship shown in Fig. 9.

Figures 10, 11 and 12 illustrate the molecular weight distributions for all standard samples, evaluated by using Sepharose CL-2B, Sepharose CL-6B and Sephadex G-75, respectively. Since molecular weight can be estimated by gel filtration only within the range of M_w for the samples used in the calibration, molecular weight distributions for the semi-standard samples, P-1200 and P-3, were not evaluated; however, elution patterns for these samples indicate that the distributions may

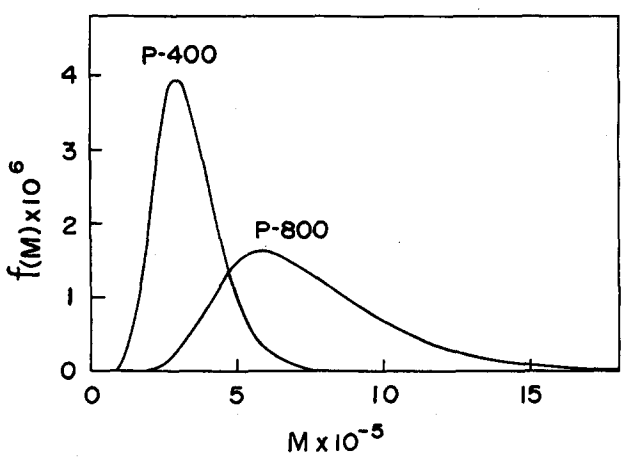

Fig. 10. Molecular weight distributions of pullulan standard samples P-800 and P-400 obtained from gel filtration with Sepharose CL-2B.

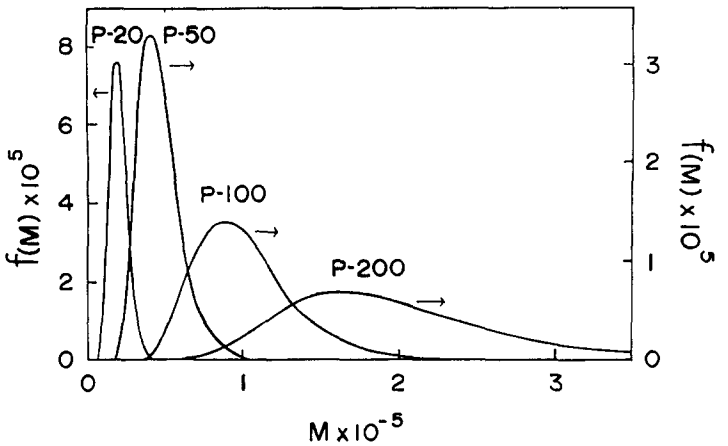

Fig. 11. Molecular weight distributions of pullulan standard samples P-200, P-100, P-50 and P-20 obtained from gel filtration with Sepharose CL-6B.

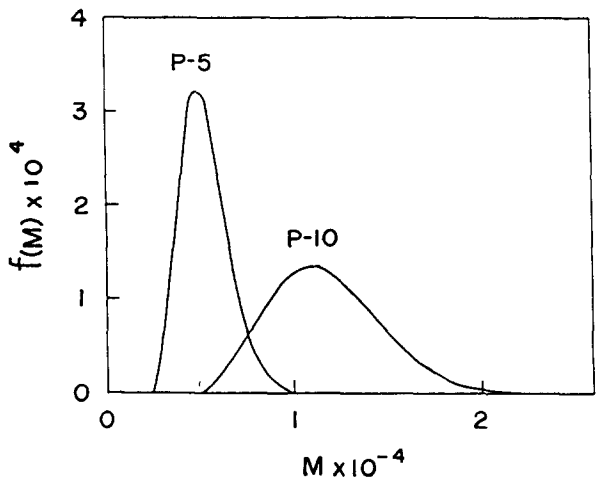

Fig. 12. Molecular weight distributions of pullulan standard samples P-10 and P-5 obtained from gel filtration with Sephadex G-75.

be as narrow as those of the standard samples so they can be used for the calibration of the gel columns.

The values of M_n, M_w, M_z, M_w/M_n and M_z/M_w were calculated from the distribution curves and summarized in Table 3, together with those

TABLE 3
Molecular Weights and Heterogeneities of Pullulan Standard Samples Evaluated by Gel Filtration and Sedimentation Equilibrium

	P-800	*P-400*	*P-200*	*P-100*	*P-50*	*P-20*	*P-10*	*P-5*
$M_n \times 10^{-4}$								
gel[a]	65·2	30·3	17·4	9·2	4·34	1·96	1·10	0·50
$M_w \times 10^{-4}$								
gel	75·8	33·7	19·5	10·0	4·70	2·09	1·17	0·53
sed[b]	75·8	33·8	19·4	9·5	4·67	2·08	1·20	0·53
$M_z \times 10^{-4}$								
gel	88·7	37·1	21·7	11·0	5·09	2·24	1·24	0·56
sed	90·2	39·0	20·6	10·9	5·28	2·18	1·30	0·55
M_w/M_n								
gel	1·16	1·11	1·12	1·10	1·08	1·07	1·06	1·06
M_z/M_w								
gel	1·17	1·10	1·11	1·10	1·08	1·07	1·06	1·06
sed	1·19	1·15	1·06	1·15	1·13	1·05	1·08	1·04

[a] By gel filtration.
[b] By sedimentation equilibrium.

observed by sedimentation equilibrium. As would be expected the values of M_w calculated from gel filtration measurements and determined by sedimentation equilibrium are in good agreement. Whereas it is appropriate to use the values of M_w determined by the absolute method of sedimentation equilibrium to describe the fractions, M_w/M_n or M_z/M_w evaluated by gel filtration should be used as a measure of heterogeneity. As previously discussed M_z/M_w obtained by sedimentation equilibrium may be less reliable.

The values of M_w/M_n are similar to those for M_z/M_w and are close to or smaller than 1·1 except for P-800. It follows that these pullulan samples are well fractionated and can be used as a series of standard polysaccharide samples with narrow molecular weight distributions.

The molecular weight distribution of P-800 is broader than those of the other samples. This may be due to difficulties in the fractionation of high molecular weight pullulans. The ratio for M_w/M_n of 1·16, however, is still fairly small and P-800 may also be used as a standard sample of high molecular weight.

CONCLUSIONS

The major results from this present study are as follows:

1. Pullulan samples, except those of very high molecular weight, are fairly stable in pure water.
2. In aqueous solution, pullulan with a molecular weight above 2×10^4 has the characteristics of a random coil polymer in a good solvent and shows no anomalous behaviour.
3. Eight pullulan samples with M_w determined by sedimentation equilibrium ranging from 5×10^3 to 8×10^5 were prepared. These samples have a very narrow molecular weight distribution (M_w/M_n close to or smaller than 1·1).

It is concluded that the pullulan samples prepared in this work may be used as a series of standard polymers not only for the calibration of chromatographic methods but also for various other studies on water-soluble polymers.

Further measurements of the molecular weight and the molecular weight distribution of these pullulan samples are in progress by various methods in several laboratories in Japan and the results will be reported later.

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