Size Distribution of Dispersing Units in Corn Amylose[†]

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As the first step to reveal the size distribution of dispersing units in amylose, an amylose sample from corn starch was subjected to gel-filtration chromatography on columns of Sephadex G 200 and Sepharoses 6 B, 4 B and 2 B under protection of the sample from retrogradation by the use of thiocyanate in the solvent system in the chromatography.

A considerable amount of dispersing units with unexpectedly large size was detected in the amylose sample as a fraction which was excluded from gels of Sepharoses, though the size distribution was considered to be rather continuous covering the units with smaller size of the regular amylose in the sample. The excluded fraction involved an appreciable amount of aggregates of amylose but no usual amylopectin. Nevertheless, the fraction involved some α -1,6 glucosidic linkages susceptible to isoamylase. The digest of the fraction by the enzyme gave a product which showed size distribution similar to that of the regular size of amylose in the original sample. Both the digested product and the regular amylose had much larger size in molecular weight than the digest of amylopectin by the same enzyme.

The results indicate that the corn amylose sample is a mixture of the units with regular size amylose and those with very large one, the latter having various numbers of branches with the size of regular amylose. Thus, it is considered that the size of dispersing units in the corn amylose sample is characterized as a pattern of such continuous distribution ranging from few hundred thousand up to more than several million in terms of molecular weight.

In the characterization of starches from various origins, the content of amylose has been considered to be one of the important factors. The reason is that amylose shows a peculiar behaviour of retrogradation from aqueous dispersion, though it is rather the minor component in regular starches. Amylose is practically distinguished from amylopectin, the major component of starch, by its specific properties to give a deep blue reaction with iodine and to form precipitate with *n*-butanol making a complex.

Since such properties of amylose are usually

attributed to the linear structure of the molecule in contrast to the finely branched amylopectin molecule, not only the content but also the size of amylose molecule is considered to be another important factor in determining the contribution of amylose to the nature of a starch paste. Thus, the size of amylose from various origins was estimated as the mean molecular weight between 3×10^5 and 6×10^5 , and the estimated value has been considered as the regular size of amylose.¹⁻³⁾ Such mean molecular weight, however, has given little information for the characteriza-

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1) R. L. Whistler and G. H. Hilbert, *J. Am. Chem. Soc.*, **67**, 1161 (1945).

2) R. W. Kerr and F. C. Cleveland, *ibid.*, **74**, 4036 (1951).

3) C. T. Greenwood, *Stärke*, **12**, 169 (1960).

tion of starches because of the lack of information about the size distribution of the dispersing units in amylose of each starch. Everett and Foster⁴ measured light scattering of subfractions obtained from potato amylose by the fractional precipitation with ethanol and suggested that the molecular size distributed from 1.5×10^5 up to 2.5×10^6 . Cowie^{5,6} also suggested essentially the same size distribution ranging from 1.2×10^5 to 4.8×10^6 in potato amylose from measurements of light scattering and viscosity of subfractions obtained by the fractional precipitation method.

In the present work, as the first step for the direct observation of the size distribution of dispersing units in amylose, an amylose sample from corn starch was subjected to gel-filtration chromatography on columns of Sephadex G 200 and Sepharoses 6 B, 4 B and 2 B under protection of the sample from retrogradation by the use of thiocyanate in the solvent system in the chromatography.

MATERIALS AND METHODS

Preparation of an amylose sample from corn starch.

A sample of amylose was prepared from commercial corn starch according to the procedure described by Gilbert *et al.* for the preparation from potato starch.⁷ In the present case, granules of corn starch were swollen in a 0.20 N sodium hydroxide solution for 15 min, while potato starch was treated in 0.14 N of the alkali for 5 min. Under the present conditions of alkali treatment, granules of corn starch are swollen and most of amylose in the granules is dispersed into the medium as reported in the previous report.⁸

To 270 ml of the aqueous suspension containing 4 g of corn starch was added 30 ml of 2.0 N sodium

hydroxide solution under gentle stirring at room temperature (23°C). After standing for 15 min to complete swelling of the starch granules, 80 ml of 5% sodium chloride solution was added, and then the alkaline paste was neutralized with hydrochloric acid. The granular gels of the swollen starch were removed by centrifugation at $2,600 \times g$ for 20 min. From the clear supernatant, amylose was precipitated as a complex with *n*-butanol by saturating it with the alcohol at room temperature. The precipitate was suspended in an aqueous solution saturated with *n*-butanol and then centrifuged. The precipitate of amylose-*n*-butanol complex was stored as a suspension in the saturated butanol solution.

Dispersion of waxy corn starch. Commercial waxy corn starch was suspended in deionized water to 1% and dispersed by heating at 125°C for 2 hr under pressure.

Isoamylase. Isoamylase from *Escherichia intermedia* was kindly supplied from Professor S. Ueda, Kyushu University. This preparation had isoamylase activity of 440 units* per mg of dry matter and no α -amylase activity was detected. This enzyme can hydrolyze α -1,6 glucosidic linkages in amylopectin molecules even when the branching moiety is as small as maltosyl or maltotriosyl group.⁹

Gel-filtration chromatography. Sephadex G 200, Sepharoses 6 B, 4 B and 2 B (Pharmacia Fine Chemicals, Uppsala, Sweden) were employed as the gel materials for the present gel-filtration chromatography. The exclusion limits of these materials for polysaccharides are, according to the producer's indication, 2×10^6 , 1×10^6 , 5×10^5 and 2×10^7 , respectively. The solvent system was 0.05 M phosphate buffer (pH 6.9) containing potassium thiocyanate in a definite concentration.

9) S. Ueda and N. Nanri, *Appl. Microbiol.*, **15**, 429 (1967).

* An isoamylase unit is defined as an increment in optical extinction at 620 m μ of 0.01 in 1 hr at 40°C under the following conditions.⁹ The reaction mixture consisted of 5 ml of 1% soluble glutinous rice starch solution, 1 ml of 0.5 M acetate buffer (pH 6.0), and 1 ml of enzyme solution. After incubation at 40°C for 1 hr, 1 ml of the reaction mixture was withdrawn and mixed with 1 ml of 0.01 N iodine solution. Water was then added to a total volume of 25 ml, and the optical extinction was measured with a photocell of 1 cm wide.

4) W. W. Everett and J. F. Foster, *J. Am. Chem. Soc.*, **81**, 3459 (1959).

5) J. M. G. Cowie, *Makromol. Chem.*, **42**, 230 (1961).

6) J. M. G. Cowie, *J. Polymer Science*, **49**, 455 (1961).

7) L. M. Gilbert, G. A. Gilbert and S. P. Spragg, "Methods in Carbohydrate Chemistry," Vol. IV, ed. by R. L. Whistler, R. J. Smith and J. N. BeMiller, Academic Press Inc., New York, N. Y., 1964, p. 25.

8) S. Sato, S. Oka and S. Shigeta, *Agr. Biol. Chem.*, **33**, 1134 (1969).

The gel material was swollen and equilibrated with the buffer, then packed into a column of 2 cm in diameter and 25 cm in height. The column was washed with more than 500 ml of the buffer to stabilize the gel bed. The total volume of the solvent in the gel bed, V_t , was determined from the elution volume required to elute glucose under the chromatographic conditions, and the void volume, V_o , was determined from the elution volume of the first peak of Blue Dextran 2000 (Pharmacia Fine Chemicals, Uppsala, Sweden). Thus, the elution volume of a sample, V_e , was standardized by the index of K_d according to equation (1).

$$K_d = (V_e - V_o) / (V_t - V_o) \quad (1)$$

Onto the column, 1 ml of a sample dispersed in the buffer containing potassium thiocyanate was applied, and chromatographed with the same buffer at the flow rate of 40~60 ml/hr. Portions of 2.5 ml of the effluent were collected, and the carbohydrate content in every fraction was determined by the phenol-sulfuric acid method.¹⁰⁾

For the convenience of comparison of chromatographic patterns from different columns, chromatograms were usually given as plots of the quantity of carbohydrate in the fraction versus the corresponding K_d value.

RESULTS

Change in elution pattern of corn amylose in gel-filtration chromatography by difference in size of network in gel material

Fifteen milligram portions of the corn amylose sample were chromatographed on columns of Sephadex G 200 and Sepharoses 6B, 4B and 2B, respectively, in the presence of 0.02 M potassium thiocyanate. In Fig. 1, the amount of carbohydrate detected in fractions is plotted versus the corresponding K_d value.

It is observed that in the case of Sephadex G 200, most of the amylose is eluted at the void volume position of the column ($K_d=0$). Judging from the exclusion limit of the gel material, the dispersing units of the

FIG. 1. Elution Patterns of Corn Amylose in Gel-Filtration Chromatography on Columns of Gel Materials with Different Size of Network.

One milliliter portions of the aqueous dispersion containing 15 mg of the corn amylose preparation were chromatographed on columns of various gel materials.

Dimension of gel beds: 2×25 cm, solvent system: 0.05 M phosphate buffer (pH 6.9) containing 0.02 M potassium thiocyanate, flow rate: 40~60 ml/hr, volume of a fraction: 2.5 ml.

majority of the amylose are considered to have the molecular weight of more than 2×10^6 . In cases of Sepharose, however, the elution patterns give two peaks. The first peak appears at the void volume position and the second one follows. The K_d value of the second peak varies from 0.4 to 0.7 with the increase of network size in the gel material. In addition, the amount of the carbo-

10) S. Abe and H. Sakai, *Nippon Shokuhin Kogyo Gakkaishi*, **14**, 20 (1967).

hydrate eluted in the first peak decreases with the increase of the network size; one half of the amount of amylose charged on Sepharose 6B is eluted in the first peak, and the corresponding values are one fourth with Sepharose 4B, and one sixth with Sepharose 2B. The decrement in the first peak finely corresponds to the increment in the second peak. Such transformation in the chromatographic pattern indicates that the size distribution of the dispersing units in the amylose sample is not discontinuous, though the patterns in Sepharoses give two peaks.

The first peak in cases of Sepharoses indicates that the amylose sample contains considerable amount of dispersing units with very large size which is assumed to correspond to molecular weight of more than several millions from the exclusion limit of the gel materials. Since the size thus evaluated is very much larger than the usual value estimated for amylose preparations from various origins,¹⁻³⁾ three possibilities are considered; (1) a small amount of amylopectin may be contaminated in the amylose sample employed in the present experiment, (2) some amount of amylose may aggregate to form the large dispersing units, (3) some amount of unusually large size molecules of amylose may be present in the sample.

Possibility of contamination of amylopectin in the amylose sample

To examine a possible contamination of amylopectin in the amylose sample used in Fig. 1, the amylose was crystallized twice from its aqueous dispersion as the *n*-butanol complex, and the recrystallized sample was again chromatographed on a column of Sepharose 4B in the same manner as in Fig. 1.

As is seen in Fig. 2, no essential transformation is observed in the elution pattern after the recrystallization; the ratio of the area of the first peak to that of the second one, as well as the elution volumes of both peaks, does not change.

FIG. 2. Influence of Recrystallization of Corn Amylose Preparation on its Elution Pattern in Gel-Filtration Chromatography on Sepharose 4B Column.

The same corn amylose preparation as in Fig. 1 was recrystallized twice from its aqueous dispersion as *n*-butanol complex, and each recrystallized preparation was again chromatographed on the column of Sepharose 4B under the same condition as in Fig. 1.

○—○ original preparation, ●—● the first recrystallization, ●---● the second recrystallization.

On the other hand, the first peak fraction in Fig. 2 was concentrated to one tenth of the initial volume by absorbing the solvent with dry powder of Sephadex G 25. The concentrate was then subjected to the iodine test, and a deep blue color reaction was yielded.

From these facts, it is considered that the contamination of usual amylopectin in the first peak eluted from columns of Sepharoses is at least negligibly small.

Dependence of elution pattern on thiocyanate concentration

To investigate the possibility of the presence of large dispersing units formed by aggregation of amylose molecules, the influence of the thiocyanate concentration on the elution pattern of the amylose sample from Sepharoses was examined.

It is seen in Fig. 3 that when the salt concentration is raised from 0.02 M to 0.1 M, the area under the first peak decreases approximately to one half or to one third. However,

FIG. 3. Dependency of Elution Pattern of Corn Amylose Preparation in Gel-Filtration Chromatography on Concentration of Thiocyanate in Solvent System.

One milliliter portions of the same amylose dispersion as in Fig. 1 were chromatographed on columns of three Sepharoses as indicated with the buffer containing 0.02 M, 0.1 M and 1.0 M potassium thiocyanate. Other conditions for chromatography were the same as those in Fig. 1.

---- 0.02 M KSCN, ●—● 0.1 M KSCN, ○—○ 1.0 M KSCN.

no more decrease in the area is observed even when the salt concentration is raised up to 1.0M. Thus, the first peak in the presence of 0.1 to 1.0M of the salt suggests the presence of a large size dispersing unit which is not dissociated by the salt.

Susceptibility of large size dispersing units to isoamylase

To detect the contamination of amylopectin or the presence of some α -1,6 glucosidic linkages in the above mentioned large size dispersing units involved in the amylose sample, the fraction at the first peak on the Sepharose 4B chromatogram was further treated with isoamylase at pH 6.0 and 47°C for 0.5 and 2 hr in the ratio of 6 units of the enzyme per 1 mg of the carbohydrate. The reaction mixture was then concentrated by means of dry Sephadex G 25 and chromatographed on the Sepharose 4B column under the same condi-

FIG. 4. Susceptibility of Dispersing Units with Very Large Size in Corn Amylose Preparation to Isoamylase.

The first peak fraction on the Sepharose 4B chromatogram was further treated with isoamylase at pH 6.0 and 47°C for 0.5 and 2 hr in the ratio of 6 units of the enzyme per 1 mg of carbohydrate. The reaction mixture was then concentrated by means of dry Sephadex G 25 and chromatographed on Sepharose 4B column under the same conditions as that in Fig. 1. For comparison, a sample of waxy corn starch was also treated.

●—● 0.5 hr treatment, ○—○ 2 hr treatment, ---- waxy corn starch, ×—× waxy corn starch after 0.5 hr treatment.

tion as that in Fig. 1. For comparison, the dispersion of waxy corn starch was also treated in the same manner as above.

In Fig. 4, the isoamylase digest of the first peak fraction gives a large and broad peak around the position of $K_d=0.5$. This is similar to that of the second peak of the original amylose sample observed in Figs. 1~3. In this case, the enzymic treatment must be enough for completing the digestion because of the good coincidence of the chromatograms of 0.5 and 2 hr treatments. On the other hand, the digest of waxy corn starch, amylopectin, gives the main peak at the position of $K_d=0.9$. The difference in K_d value means that the size of dispersing units in the digest of the first peak fraction distributes in a much higher level of molecular weight than that of

amylopectin, though both samples are susceptible to isoamylase.

From the results, it is considered that the very large dispersing units in the amylose sample consist of some subunits which are linked together through α -1,6 glucosidic linkages forming such very large units. However, it is also considered that the size of the subunits is much larger than that of amylopectin and distributes in essentially the same level as that of the second peak fraction in the Sepharose 4 B chromatogram which is the major fraction of the original amylose sample.

DISCUSSION

A considerable amount of dispersing units with unexpectedly large size was detected in the amylose sample from corn starch as the fraction which was excluded from gels of Sepharoses in gel-filtration chromatography in the presence of potassium thiocyanate, though the size distribution of the dispersing units was considered to be rather continuous covering the units with smaller size in the major part of the sample (Fig. 1). The excluded fraction involved an appreciable amount of aggregates of amylose but no amylopectin (Figs. 2 and 4). Nevertheless, the fraction contained some α -1,6 glucosidic linkages susceptible to isoamylase. The digest of the fraction by the enzyme gave products which had a size distribution similar to that of the smaller size units in the original sample. However, both the digested product and the smaller size units in the amylose had a much larger size in molecular weight than the digest of amylopectin by the same enzyme (Fig. 4).

The size of dispersing units in amylose has been usually estimated to be from 3×10^5 to 6×10^5 in terms of mean molecular weight.¹⁻³¹ These values correspond to the size of most of the dispersing units with smaller size in the amylose sample, because the values correspond to the value of K_d around 0.5 in the Sepharose 4 B chromatogram. Thus, the

size of dispersing units in the fraction excluded from the gels of Sepharoses is roughly estimated to be in the region of more than several million in terms of molecular weight. The fractions with the highest values of molecular size estimated by Everett and Foster⁴¹ and Cowie^{5,61} probably correspond to that excluded from Sepharoses in the present work.

On the other hand, Kjölberg and Manners¹¹⁾ had suggested the presence of slightly branched molecules in amylose from the observation that incubation of *amylose β -limit dextrin* with yeast isoamylase caused an increase in β -amylolysis limit with a limited fall in specific viscosity. Later, Banks and Greenwood¹²⁾ concluded from results of experiments with pullulanase¹³⁾ that some α -1,6 glucosidic linkages were present in amyloses of various origins and that these formed the branched amylose molecules. In the molecules, long amylose chains branched through α -1,6 glucosidic linkages with intervals of several hundred of glucose residues. The branched amylose is also considered to correspond to the large size units in the present work.

Accordingly, the corn amylose sample is considered to be a mixture of the units with regular size and those with very large one, the latter having various numbers of branches with the size of regular amylose. Thus, the size of dispersing units in the corn amylose sample is characterized as a pattern of such continuous distribution ranging from few hundred thousand up to more than several million in terms of molecular weight.

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11) O. Kjölberg and D. J. Manners, *Biochem. J.*, **84**, 50 p (1962); *ibid.*, **86**, 258 (1963).

12) W. Banks and C. T. Greenwood, *Arch. Biochem. Biophys.*, **117**, 674 (1966); *Stärke*, **7**, 197 (1967).

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