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PURIFICATION AND STRUCTURE OF AMYLOSE FROM RICE STARCH

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ABSTRACT

A method for the purification of amylose from rice starch has been developed. The critical step was the removal of insoluble amylopectin-like contamination from crude amylose in water at 50° by ultracentrifugation at 100,000*g* for 1 h. The conventional autoclaving procedure was avoided. Gel-permeation chromatography and h.p.l.c. indicated the amylose specimens prepared from indica and japonica rice starches to be pure. The iodine affinities and blue values of the specimens were 20.0–21.1 g/100 g and 1.40–1.47, respectively. The amyloses had number-average d.p. ($\overline{d.p.}_n$) values of 980–1110, average chain-lengths of 250–370, and beta-amylolysis limits of 73–84%, indicating them to be slightly branched molecules with 2–5 chains on average. The weight-average d.p. ($\overline{d.p.}_w$) values were 2750–3320, and the high $\overline{d.p.}_w/\overline{d.p.}_n$ values (2.6–3.4) suggested broad distributions of molecular weights.

INTRODUCTION

Detailed elucidation of the branched structure of amylose[1,2] requires pure specimens from various origins. Takeda *et al.*[2] have reported that gel-permeation chromatography is more sensitive for estimating the contamination of amylose by a small amount of amylopectin than the usual iodine-affinity determination. They obtained chromatographically pure specimens of amylose from some root and tuber starches, but not from cereal starches, and characterised them in terms of molecular weight, chain length, number of chains per molecule, and molecular-weight distribution[1–3]. Some relationships between their structures and functional properties were also reported[4,5].

We now report on the molecular structures of amyloses from indica and japonica rice starches. Hizukuri *et al.*[6] reported that the average molecular weights of some indica rice amyloses were appreciably smaller than those of tuber and root amyloses. However, the starch was dispersed in water by autoclaving at 125° prior

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to fractionation of its components and this treatment could account for the small molecular weight. Autoclaving is usually considered to be a requisite for fractionation of cereal starches due to the tight association of their molecules, but it is difficult to prepare pure amylose from rice starch by this procedure. Therefore, we have developed a method which avoids conventional autoclaving.

MATERIALS AND METHODS

Preparation of rice amylose. — Rice starch (10 g, dry weight) was dissolved in methyl sulfoxide (300 mL) by heating at ~100° with stirring under nitrogen, and ethanol (300 mL) was then added to the solution. The mixture was stored at ~0° for a few hours, and the precipitate was collected by centrifugation (2500*g*, 20 min, room temp.) and redissolved in methyl sulfoxide (300 mL). The starch, after repeated precipitation with ethanol, was dispersed in water (400 mL) at 70–80°, and a mixture of 1-butanol (100 mL), 3-methyl-1-butanol (100 mL), and water (1300 mL) was added. The dispersion was stirred and boiled under reflux for 3 h under nitrogen, cooled to 50°, and kept in a Styrofoam box overnight at room temperature and then at ~8° for 48 h. The precipitate was collected by centrifugation (10,000*g*, 20 min, 4°) and then suspended in aqueous 10% 1-butanol (1 L). The suspension was boiled under reflux for 1 h, cooled, and stored at ~8° for 24 h. The precipitate (crude amylose) obtained by centrifugation was dispersed in water (450 mL) at 80–90° under nitrogen, and then immediately centrifuged (100,000*g*, Hitachi model 65P) with a rotor (RP-42) prewarmed at 50°. To the supernatant solution was added water (550 mL) at ~70° and 1-butanol (100 mL). The solution was boiled under reflux for 10 min, then filtered through a glass filter (G-5), boiled under reflux for a few min, and cooled (1st recrystallisation). The precipitate obtained by centrifugation was suspended in aqueous 10% 1-butanol (1 L), and the suspension was again boiled under reflux. These procedures were repeated twice (2nd and 3rd recrystallisations). The amylose–1-butanol complex was collected by centrifugation and ground with ethanol, and the amylose was collected on a glass filter (G-2), washed with ethanol and ether, and then dried *in vacuo* at room temperature over $CaCl_2$. The amylose specimens (~1.5 g), which were stored as dry powders rather than as the wet amylose–1-butanol complex in order to avoid variable degradation, were stable and dissolved instantaneously in a dilute alkaline solution to give clear solutions. These solutions before and after neutralisation gave reproducible results for the chemical and physical analyses. The precipitates (0.2–0.3 g) obtained on ultracentrifugation were also dried.

Materials. — Indica rice starches were prepared from milled rice flour by exhaustive extraction of protein with sodium dodecyl benzenesulfonate[7], and japonica rice starches from rice grains by alkaline extraction of protein. The polished grains were ground in aqueous 0.3% NaOH after soaking them for 3 days. The slurry was squeezed through bleached cotton cloth, and the starch was extracted repeatedly with aqueous 0.3% NaOH until the extract became negative

to the biuret test. The starch was washed repeatedly with water until the pH of the supernatant solution became neutral, and then air-dried.

Beta-amylase was prepared[8] from sweet potato and recrystallised from aqueous ammonium sulfate to improve its stability during storage. Toyopearl HW-75F was obtained from Toyo Soda Manuf. Co. Ltd. (Tokyo).

Methods. — The purity of amylose specimens was examined by gel-permeation chromatography[2] on Toyopearl HW-75F. Iodine affinity was determined at 25° by amperometric titration[9]. The blue value was determined by the method described elsewhere[5]. The number-average d.p. ($\overline{d.p._n}$) of amylose was determined by the modified Park–Johnson method[1]. The weight-average d.p. ($\overline{d.p._w}$) and its distribution were determined by high-performance gel chromatography on three sequentially linked columns (TSK-GEL G6000PW, G4000PW, and G3000PW; Toyo Soda Manuf. Co. Ltd.) using a Toyo Soda 803C chromatograph with a low-angle laser-light-scattering photometer (Toyo Soda LS-8) and a differential refractometer (Toyo Soda RI 8010) as detectors[3,10]. The average chain-length ($\overline{c.l.}$) was determined by the rapid Smith-degradation method[1] with minor modification. The amylose solution was adjusted[2] to pH 5. The number of chains per molecule is $\overline{d.p._n}/\overline{c.l.}$ The beta-amylolysis limit was determined method by a described[2]. Phosphate was determined[11] as inorganic phosphate after treatment with hot perchloric acid[12]. Carbohydrate was determined by the anthrone–sulfuric acid method[13]. The limiting viscosity number $[\eta]$ was determined in M KOH with an Ostwald viscometer at 22.5°. The treatment of amylose with *Pseudomonas* isoamylase was carried

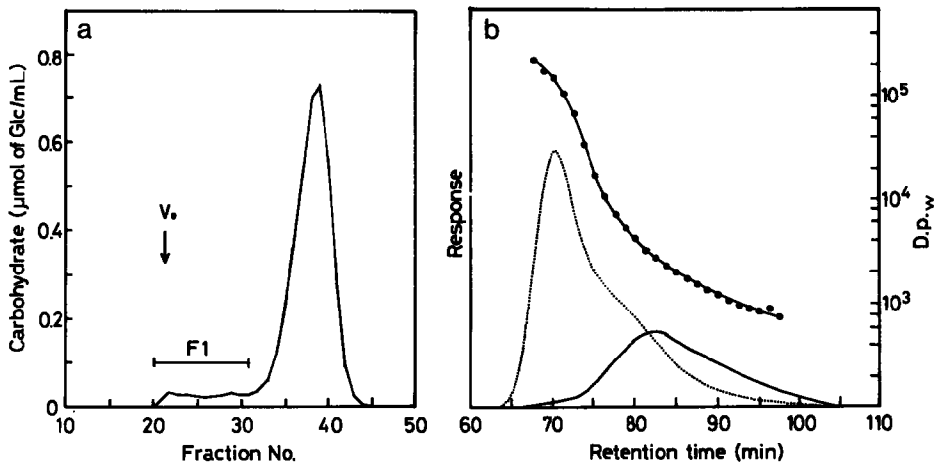

Fig. 1. Gel filtration (a) and h.p.l.c. (b) elution curves for an IR42 amylose purified by 12 recrystallisations. Gel filtration (10-mL fractions) was performed with a column (2.6 × 100 cm) of Toyopearl HW-75F and the other conditions were as reported[2]. The void volume of the column was determined from the elution volume for potato amylopectin. H.p.l.c. elution curves were monitored with a low-angle laser-light-scattering photometer (------) and a differential refractometer (——)[3]. The elution solvent was 0.1M sodium phosphate buffer (pH 6.1) containing 0.02% of sodium azide at 0.495 mL/min, and 1 mL of amylose solution (0.3 mg/mL) was injected; ●——●, $d.p._w$.

out under the described conditions[1]. Incubations for 1.5 and 2.5 h resulted in the same extent of hydrolysis, and the enzyme was still active after incubation.

RESULTS AND DISCUSSION

Lipid in cereal starches appears to inhibit granular swelling and molecular dispersion when an aqueous suspension is heated[14,15] and thus interferes with fractionation of amylose and amylopectin. Therefore, removal of lipid was attempted for effective fractionation under mild conditions, that is, extraction with aqueous 85% methanol, aqueous 80% 1,4-dioxane, and water-saturated 1-butanol. However, these procedures were not satisfactory, judging from the clarity of the paste, and the best procedure involved repeated dissolution in hot methyl sulfoxide and precipitation with ethanol. A similar treatment with cold methyl sulfoxide has been applied to some other cereal starches[16,17]. The defatted, indica rice starch (IR42) was fractionated by dispersal in an aqueous mixture of 1-butanol and 3-methyl-1-butanol with heating and cooling. The crude amylose thus obtained was purified by 12 recrystallisations from hot, aqueous 10% 1-butanol. The iodine affinity (19.2 g/100 g) and blue value (1.28) of the product seemed to be a little lower than those of pure amylose[2]. Gel-permeation chromatography of the amylose on Toyopearl HW-75F (Fig. 1a) gave a main peak and a minor flat peak (F-1) that was eluted in the void volume, suggesting the presence of amylopectin because partially purified lily amylose showed a similar minor peak (F-1) for amylopectin[2]. The h.p.l.c.-elution curves and the semilogarithmic relationship between the retention time and $\overline{d.p.}_w$ (Fig. 1b) confirmed that the specimen contained material of higher molecular size with a lower hydrodynamic volume, probably amylopectin because logarithmic plots of $\overline{d.p.}_w$ gave a straight line over the entire elution range for pure amylose

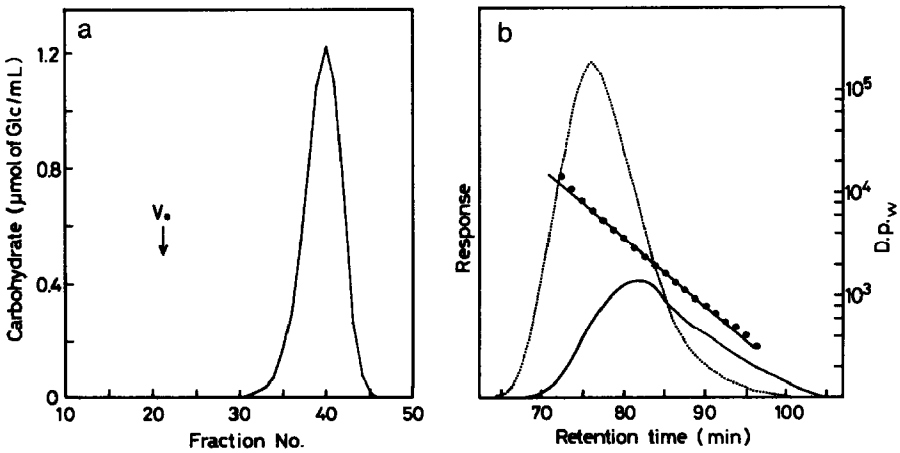

Fig. 2. Gel filtration (a) and h.p.l.c. elution (b) curves for IR42 amylose purified by ultracentrifugation and 3 recrystallisations. For h.p.l.c., 1 mL of amylose solution (0.4 mg/mL) was injected and the other conditions were as in Fig. 1: ------, low-angle laser-light-scattering photometer response; ——, differential refractometer response; ●——●, $d.p._w$.

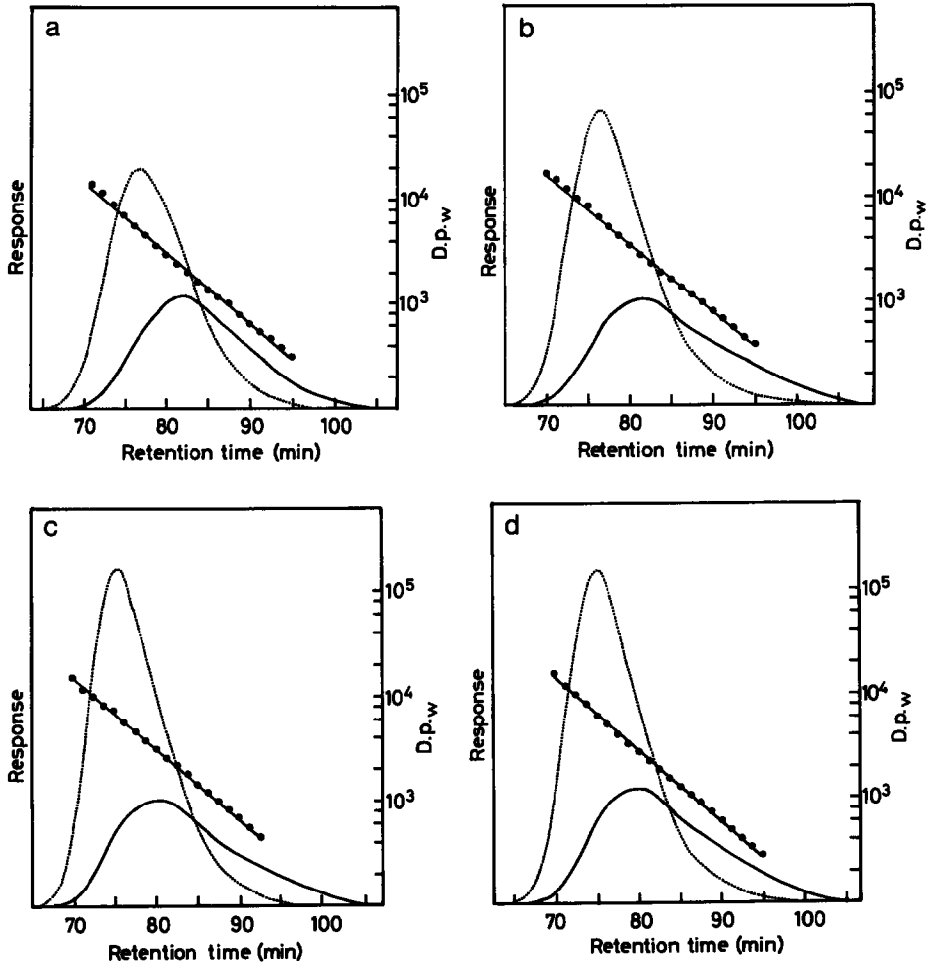

Fig. 3. H.p.l.c. elution curves for amyloses IR32 (a), IR36 (b), Hokkaido (c), and Sasanishiki (d); 1 mL of amylose solution (0.4 mg/mL) was injected: ------, low-angle laser-light-scattering photometer response; ——, differential refractometer response; ●——●, $d.p._w$.

TABLE I

PROPERTIES OF PRECIPITATES OBTAINED ON ULTRACENTRIFUGATION OF CRUDE AMYLOSES DISPERSED IN HOT WATER

Property	*IR32*	*Sasanishiki*
Blue value	0.37	0.27
λ_{max} (nm)	606	600
C.I.	32.3	31.1
Beta-amylolysis limit (%)	64	62

with this column system. F-1, which was ~6% of the specimen, had a blue value of 0.439, a $\overline{c.l.}$ of 26.5, and a beta-amylolysis limit of 63%, indicating it to be either amylopectin containing a small proportion of amylose or an intermediate component since the values were higher than those of normal amylopectin[4,5]. F-1 was not studied further. A similar amount of F-1 was obtained from IR42 amylose recrystallised 6 and 10 times, and from specimens of some other rice varieties. It was concluded that repeated recrystallisation does not purify rice amylose, although it is useful for root and tuber amyloses[2]. Further repeated defatting of the starch prior to fractionation did not remove F-1, but the following procedure was successful.

The crude amylose gave a faintly opaque solution in hot water and the insoluble material was removed by ultracentrifugation (100,000*g*, 1 h, ~50°). This procedure was advantageous since recrystallisations then gave chromatographically pure amylose (IR42, Fig. 2a). The h.p.l.c.-elution profiles and $\overline{d.p.}_w$ curve of the specimen (Fig. 2b) showed that there was a linear relationship between the retention time and log of $\overline{d.p.}_w$, and that there was no material of high molecular size as observed in Fig. 1b. The specimens of rice amylose from other indica varieties, IR32 and IR36, and japonica varieties, Hokkaido (unknown variety) and Sasanishiki, thus prepared were pure by gel-permeation chromatography (chromatograms not shown) and h.p.l.c. (Fig. 3). Thus, ultracentrifugation is useful for the purification of rice amylose and, probably, other cereal amyloses.

The amounts of precipitate on ultracentrifugation were 2–3% of the starches. The properties of the precipitates from the IR32 and Sasanishiki varieties (Table I) indicated them to be either amylopectin containing a small proportion of amylose or an intermediate component since the blue value, λ_{max}, $\overline{c.l.}$, and beta-amylolysis limit were a little higher than those of amylopectin. Their properties were very similar to those of F-1 in Fig. 1a. The non-removal of F-1 by the repeated recrystallisation may be due to the tight intermolecular association.

The rice amyloses examined were prepared from starches extracted by two different methods using a surfactant and a dilute alkaline solution. However, this difference in procedure would not affect the molecular properties of amylose since corn starches prepared by using water at 8 and 52°, and dilute alkali (room temperature), gave amyloses having the same molecular weight[18]. Conceivably, the alkaline solution may degrade amylose in solution, but not in starch granules at room temperature. Table II summarises the properties of rice amyloses. These specimens had iodine-affinity values of 20.0–21.1 g/100 g, blue values of 1.40–1.47, and λ_{max} 653–658 nm. The rice starch contained phosphorus (10–20 p.p.m.) attached to glucosyl residues, the values being lower than those of root and tuber starches[19]; the rice amyloses were almost free from phosphorus (<1 p.p.m.), in contrast to root, bulb, and tuber amyloses which had detectable amounts (2–9 p.p.m.)[2,5]. The limiting viscosity numbers $[\eta]$ of the rice amyloses were in the range 180–208 mL/g (22.5°), which is higher than those (80.4–177 mL/g at 30°) for other indica rice varieties[6,20]. These $[\eta]$ values were similar to those (202, 228 mL/g) of kuzu amylose[2,4] and lower than those of lily (312 mL/g)[5], tapioca (384 mL/g), and potato

TABLE II

PROPERTIES OF RICE AMYLOSE

Property	*Indica*			*Japonica*	
	IR32	*IR36*	*IR42*	*Hokkaido*[a]	*Sasanishiki*
Iodine affinity (g/100 g)	20.3	21.1	20.0	20.3	20.6
Blue value	1.43	1.45	1.40	1.47	1.40
λ_{max} (nm)	657	653	653	656	658
Limiting viscosity number (mL/g)	180	208	192	208	216
$\overline{D.p.}_n$	1040	920	980	1100	1110
C.I.	250	370	230	260	320
Number of chains per molecule	4.2	2.5	4.3	4.2	3.5
Beta-amylolysis limit (%)	73	84	76	77	81
Beta-amylolysis limit, with pullulanase (%)[b]	100	101	101	102	103

[a]Unknown variety from Hokkaido. [b]The conditions of simultaneous hydrolysis with pullulanase were the same as in ref. 2.

TABLE III

$\overline{D.P.}_w$ AND APPARENT DISTRIBUTION OF D.P.$_w$ FOR RICE AMYLOSE

Degree of polymerisation	*Indica*			*Japonica*	
	IR32	*IR36*	*IR42*	*Hokkaido*	*Sasanishiki*
$\overline{D.p.}_w$	2750	2810	3320	3230	3092
$\overline{D.p.}_w$ (max)[a]	2290	2630	2720	2950	2820
$\overline{D.p.}_w/\overline{d.p.}_n$	2.64	3.05	3.39	2.94	2.79
Apparent d.p.$_w$ distribution[b]	290–8790	210–9800	260–12900	210–9860	280–9690

[a]D.p.$_w$ at the maximum of the elution peak (see Figs. 2b and 3). [b]D.p.$_w$ values of the sub-fractions (10% amylose by weight) having the lowest and highest molecular weights.

(384 mL/g) amyloses[2]. The $\overline{d.p.}_n$ of the rice amyloses from the indica and japonica varieties were in the range 920–1110, indicating that there is little variety difference in molecular weight. Lower $\overline{d.p.}_n$ values of 530–793 were determined for the amyloses prepared from autoclaved rice starches[6]. This discrepancy will be discussed later, but the molecules of the rice amyloses were certainly smaller than those of the tuber and root amyloses[2]: kuzu ($\overline{d.p.}_n$, 1540), lily (2310), tapioca (2660), and potato (4920).

Table III summarises the characteristics of the molecular size distribution determined by h.p.l.c. with monitoring by a low-angle laser-light-scattering photometer and a differential refractometer[3,10]. The $\overline{d.p.}_w$ values of the specimens, which are in the range 2750–3320, are similar to that of kuzu (3220), but about half those of potato (6360), sweet potato (5430), tapioca (6680), and lily (5010) amyloses[3]. The $\overline{d.p.}_w$ values of the rice amyloses at the point of maximum elution of carbohydrate (see Figs. 2b and 3) were 2290–2950. The ratios of $\overline{d.p.}_w/\overline{d.p.}_n$ (2.64–3.39)

are higher than those of potato (1.29), sweet potato (1.31), kuzu (2.08), lily (2.17), and tapioca (2.51) amyloses[3]. This indicates that the rice amyloses have a relatively broad distribution of molecular weights, which appears to be characteristic. The $d.p._w$ values of the 10% smallest and largest molecular sub-fractions (by weight) were in the ranges 210–290 and 8790–12900, respectively, and these amyloses showed similar distributions of $d.p._w$.

The average chain-length ($\overline{c.l.}$) of the rice amyloses differed among the varieties (Table II). The amyloses of the IR32, IR42, and Hokkaido varieties showed lower $\overline{c.l.}$ than those of the IR36 and Sasanishiki varieties. The $\overline{c.l.}$ values were about half that (670) of potato amylose, lower than that (470) of lily amylose, and similar to those of wheat (300), kuzu (320), and tapioca (340) amyloses[2]. The $\overline{d.p._n}$ and $\overline{c.l.}$ values indicate that the rice amyloses are slightly branched molecules with 2–5 chains on average, being probably mixtures of linear and branched molecules. The rice amyloses had fewer chains than tapioca (7.8) and potato (7.3) amyloses, but similar numbers of chains as kuzu and lily amyloses[2]. The variety differences among the rice amyloses in $\overline{c.l.}$ and number of chains per molecule may depend on the proportions of linear and branched molecules. The beta-amylolysis limit values of the rice amyloses were 73–84%, which were similar to those for the root and tuber amyloses[2,5]. Simultaneous hydrolysis of the rice amyloses with beta-amylase and pullulanase yielded the theoretical amount of maltose, suggesting that all of the chains involved α-(1→6) linkages. The treatment of the amyloses with *Pseudomonas* isoamylase decreased the $\overline{d.p._n}$ and increased the beta-amylolysis limit (Table IV). However, the far from complete beta-amylolysis and the number of chains of debranched amyloses indicate that isoamylase cannot cleave all of the branching linkages of rice amyloses as reported for potato amylose[1,2].

The rice amyloses of indica varieties prepared from autoclaved starches by purification with one recrystallisation showed lower $\overline{c.l.}$ values (101–157) and $\overline{d.p._n}$ values (530–793)[6]. The fewer recrystallisations without ultracentrifugation may have allowed contamination by a small proportion of amylopectin which lowered the $\overline{c.l.}$ The lower $\overline{d.p._n}$ is supposed to result from the degradation of amylose on autoclaving. The h.p.l.c.-elution curves of the IR36 amylose autoclaved at 125° in Fig. 4 reveal such degradation, because the fractions of low molecular size in-

TABLE IV

PROPERTIES OF AMYLOSES DEBRANCHED WITH ISOAMYLASE[a]

Property	*Indica*			*Japonica*	
	IR32	*IR36*	*IR42*	*Hokkaido*	*Sasanishiki*
$\overline{D.p._n}$	320	510	265	315	390
Number of chains per molecule	1.3	1.4	1.2	1.2	1.2
Beta-amylolysis limit (%)	88	88	89	86	84

[a]The conditions of isoamylolysis were the same as in ref. 1.

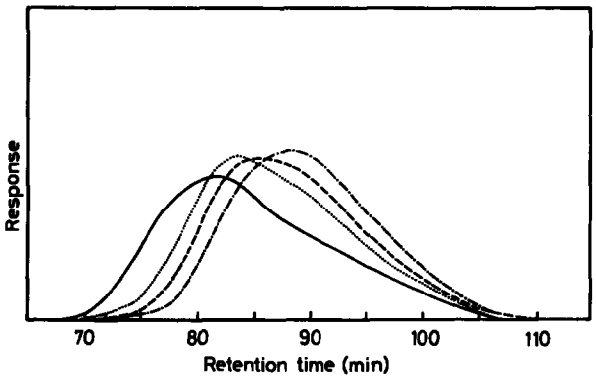

Fig. 4. H.p.l.c. elution curves with a differential refractometer for autoclaved amylose; IR36 amylose (——) at 0.4 mg/mL was autoclaved in 0.1M sodium phosphate buffer (pH 6.1) at 125° for 1 (------), 2 (– – –), and 3 h (– · –).

creased and consequently the $\overline{d.p.}_w$ markedly decreased with prolonged treatment from 2810 (original) to 1730 (1 h), 1290 (2 h), and 110 (3 h), whereas heating at 100° for 1 h scarcely decreased the $\overline{d.p.}_w$ (2680). Degradation on atmospheric heating in the aqueous mixture of 1-butanol and 3-methyl-1-butanol was negligible. Thus, autoclaving causes considerable degradation of amylose and should be avoided, and gentle boiling is recommended for fractionation and recrystallisation.

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