ELSEVIER

Carbohydrate Research 306 (1998) 421–426

CARBOHYDRATE RESEARCH

Examination of number-average degree of polymerization and molar-based distribution of amylose by fluorescent labeling with 2-aminopyridine

Isao Hanashiro, Yasuhito Takeda*

United Graduate School of Agricultural Sciences, Kagoshima University, 1-21-24, Korimoto, Kagoshima 890, Japan

Received 27 August 1997; accepted 4 November 1997

Abstract

Suitable conditions for the fluorescent labeling of the reducing residue of amylose with 2-aminopyridine were examined. Amylose of up to 38.5 nmol was labeled with a constant labeling efficiency. The same efficiencies were obtained for amyloses having a number-average degree of polymerization (dp_n) of 521–4400. The analysis of labeled amylose on size-exclusion HPLC with refractive index and fluorescence detection enabled the determination of dp_n and dp distribution on a molar basis. The analysis of eight amylose specimens from seven botanical sources (potato, sweet potato, barley, wheat, indica rice, japonica rice, and maize) gave dp_n values in good agreement with those determined by a conventional colorimetric method. The molar-based distributions of these amyloses were characteristic of botanical source and revealed the presence of several molecular species with different dp not detectable in the distribution on a weight basis. Small amyloses with a dp less than 10^3 were predominant in the cereals while amyloses with a dp over 10^3 were predominant in the tubers, suggesting a difference in the biosynthetic process determining the dp distribution of amylose between cereals and tubers.

Keywords: Starch structure; Amylose distribution; Fluorescent labeling; 2-Aminopyridine

1. Introduction

The molecular structures of amylose and amylopectin are characteristic of botanical source and affect the physicochemical properties of starch. Several parameters have been reported to express their structural characteristics [1,2], including dp distributions of (unit) chains. High performance size-exclusion chromatography (HPSEC) with refractive index (RI) detection is most convenient for the analysis of the distributions because carbohydrate has no functional group suitable for other on-line determination. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD) has been also

* Corresponding author. Fax: 81 + 99-285-8641; e-mail: takeda@chem.agri.kagoshima-u.ac.jp

0008-6215/98/$19.00
PII S0008-6215(97)10075-1

frequently used, especially for relatively short chains (up to a dp of about 80) [3,4]. However, the detector response of malto-oligosaccharides differs with dp [3,5] so that the resultant chromatogram is presented on neither weight nor molar basis. Recently, the combination of an amyloglucosidase column reactor and HPAEC–PAD has been reported [6,7]. With this system, approximately the same detector responses were obtained for maltosaccharides with a dp of up to 7 on both weight and molar bases, and it appeared to be useful to determine quantitatively malto-oligosaccharides up to dp 77 [7].

Introduction of a radioactive compound or a chromo- or fluorophore, to the reducing terminal of carbohydrate is one of the conventional techniques to examine a molar-based distribution. Takeda et al. examined the molar-based distributions of tritium-labeled amylose and side-chains of amylose [8,9]. However, the method was time-consuming because of the absence of HPLC system. Stefansson and Novotny [10], and O'Shea and Morell [11] reported fluorescent labeling and electrophoretic separation of amylose and debranched amylopectin. In these studies, however, the quantitative considerations were poor for labeling maltosaccharides with a dp higher than 8, and an acetic acid solution used [10–13] on labeling appeared to be unsuitable for amylose because of incomplete dissolution and possible hydrolysis.

In this study, we examined the labeling conditions suitable for quantitative labeling of amylose using 2-aminopyridine which was reported [14] to be useful for labeling oligosaccharides, and both dp_n and molar-based distribution of amylose were determined by HPSEC after the fluorescent labeling.

2. Experimental

Materials.—2-Aminopyridine (a special grade for fluorescent labeling) and sodium cyanoborohydride were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and Aldrich Chemical Co. Inc. (Milwaukee, WI, USA), respectively. Synthetic amyloses (AS series) were obtained from Nakano Vinegar Co., Ltd. (Aichi, Japan). Amyloses from various botanical sources were fractionated from defatted starches as 1-butanol complex and purified by ultracentrifugation as previously described [15], but potato amylose was purified only by recrystallization from 10% 1-butanol. A commercial potato amylose (type II) which was a product of Sigma Chemical Co. (St. Louis, MO, USA) was purified as described [16]. Other reagents were of the highest grade commercially available.

Fluorescent labeling of amylose.—2-Aminopyridine (2-AP) solution was prepared by dissolving 1 g of 2-AP in 760 μL of 12 M HCl [17]. Aqueous sodium cyanoborohydride ($NaCNBH_3$) was prepared just before use by dissolving 52.9 mg of $NaCNBH_3$ in 100 μL of water. Amylose was dissolved in 90% Me_2SO by heating and diluted 1.8-fold with water (up to 385 nmol/mL; for HPSEC analysis, 20 mg/mL). To 100 μL of the amylose solution in a Teflon-lined screw capped tube was added 100 μL of the 2-AP solution, the mixture was incubated at 60 °C for 1 h, 100 μL of the aqueous $NaCNBH_3$ was added, and the mixture was further incubated for 24 h. After the tube was opened under effective ventilation, the mixture was diluted to 10-fold with 2.7 mL of water, and 300 μL of 1-butanol and 3 μL of 2 M NaCl were added. Then the mixture was stored at 4 °C for at least 1 h to precipitate amylose. Excess reagents were removed by washing the precipitate with 3 mL of 10% 1-butanol containing 6 mM NaCl five times at 5 °C. The washed amylose was dissolved in 2 mL of water and the fluorescence intensity was measured (excitation, 315 nm; emission, 400 nm) with a fluorescence spectrophotometer (F-2000, Hitachi Ltd., Tokyo, Japan). For HPSEC analysis, the washed amylose precipitate was stored at 4 °C until use.

High performance size-exclusion chromatography of labeled amylose.—HPSEC was performed with a HPLC system consisting of a liquid chromatograph (HLC-803D, Tosoh Co., Tokyo, Japan), a TSK guard column PWH (7.5×75 mm), analytical columns of TSKgel G6000PW, G4000PW and G3000PW (connected in series in this order, 7.5×600 mm each, all from Tosoh), a fluorescence detector (FS-8010, Tosoh) and a refractive index detector (ERC-7512, Erma Inc., Tokyo, Japan). Excitation and emission wave lengths were 315 and 400 nm, respectively. The eluent was 0.1 M phosphate buffer (pH 6.1) containing 0.02% sodium azide and the flow rate was 0.5 mL/min. The temperature of the columns was maintained at 37 °C. The labeled amylose (2 mg) was dissolved in 50 μL of 1 M NaOH, and gradually diluted with 700 μL of water. To the amylose solution were added

200 μL of 0.5 M phosphate buffer (pH 6.1) and 50 μL of 1 M HCl, then 500 μL (1 mg of amylose) of the solution was injected after filtration through a 0.22 μm membrane filter. Dp_n of amylose was calculated from the ratio of RI response (peak area) to fluorescence response (RI/F) using a calibration line which was obtained from the RI/F values for standard amyloses (AS-110, AS-320, and AS-1000, which have dp_n of 521, 2320, and 4400, respectively, as determined by a colorimetric method [18]). The time-lag between the response profiles of the two detectors was corrected [19] by using bovine serum albumin (excitation, 280 nm; emission, 340 nm).

Other analytical methods.—Total carbohydrate and reducing residue were determined by the phenol–sulfuric acid method [20] and the modified Park–Johnson method [18], respectively.

3. Results and discussion

Labeling of amylose with 2-aminopyridine and determination of number-average dp by fluorescent labeling.—Amylose could not be dissolved completely in an aqueous solution even by heating in a boiling water bath, and at high concentration, amylose is precipitated by addition of aqueous acetic acid. Therefore, amylose was dissolved in Me_2SO and subjected to labeling with 2-AP (possible without acetic acid [17]). After removal of excess reagents, the recovery of amylose was higher than 95%. The time course of reduction with $NaCNBH_3$ showed that the residual reducing power was less than 3% after 24 h. Even after 48 h, about 2% of the residual reducing power was still detected, and the introduced fluorescence intensity increased slightly. Hence, for convenience, the 24 h reduction was performed routinely. Labeling of amylose with 8-aminonaphthalene-1, 3, 6-trisulfonic acid (ANTS) was incomplete under the standard condition (7.5% acetic acid, 37 °C, ref. [13]) with slightly decreased concentrations of ANTS and $NaCNBH_3$ (68 and 455 mM, respectively) due to increased volume by amylose solution. Moreover, increase of reducing power during incubation without the labeling reagents was observed when either the acetic acid concentration was higher than 7.5% or temperature was higher than 37 °C, due to acid hydrolysis of amylose. Therefore, labeling of amylose with ANTS could not be adopted.

The introduced fluorescence intensity (Fig. 1) was proportional to the amount of amylose up to 38.5 nmol. Labeling of amylose of higher than 38.5 nmol was impractical because of the high viscosity and uneasy handling of amylose solution. Fluorescence intensity per nmol of amylose was almost constant (868 ± 42) in the range tested. These results indicated that amylose was labeled quantitatively under the conditions described. The synthetic amyloses with different dp_n showed a linear relationship between the ratio of RI response to fluorescence response (RI/F) and dp_n value (Fig. 2), indicating no decrease in labeling efficiency with increase in dp of amylose. Hence, the dp_n of amylose specimens can be determined from an RI/F value calculated from the peak area using the calibration line obtained from amyloses with known dp_n. Plots for eight amyloses from botanical source (Fig. 2) showed a linear relationship with a correlation coefficient of 0.996 and well fit

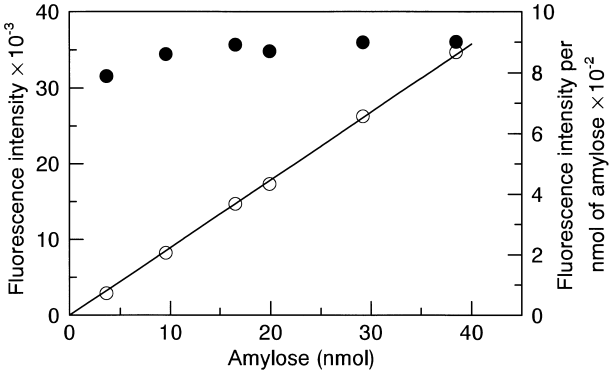

Fig. 1. Relationship of introduced fluorescence intensity to amount of amylose: ○, fluorescence intensity; ●, fluorescence intensity per nmol of amylose. Amylose with dp_n of 796, purified from commercial potato amylose, was used.

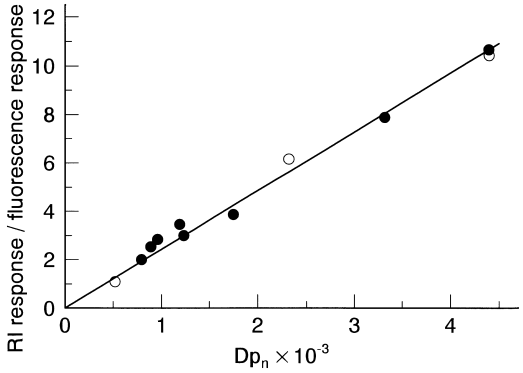

Fig. 2. Relationship between the ratio of RI response to fluorescence response and dp_n determined by HPSEC and a colorimetric method: ○, synthetic amylose; ●, amylose from botanical source.

Table 1
Dp_n and apparent dp distributions of amyloses

Source	Dp_n determined by		Apparent dp distribution[a]
	Labeling method	Colorimetric method	
Potato	4370	4400	9770–970
Sweet potato	3230	3320	7920–440
Barley	1580	1750	3880–260
Wheat	1220	1230	3130–190
Rice (indica)	1410	1190	3100–290
Rice (japonica)	1150	963	2750–230
Maize	1030	891	2370–230
Potato (commercial)	807	796	1730–220

[a] The dp values of the elution positions at which 10 and 90% of amylose in mol were eluted.

with the calibration line obtained from the synthetic amyloses. The dp_n was determined from the RI/F value, and compared with the dp_n obtained by the colorimetric method (Table 1). These values were in good agreement although maize and rice amyloses had a dp_n slightly higher than that obtained by the colorimetric method. The RI/F values for these amyloses were reproducible (RSD < 5.5%).

Characteristics in molar-based distributions of amyloses from various sources.—The profiles (Fig. 3) obtained by fluorescence and RI detection correspond to molar- and weight-based distributions, respectively. The molar-based distribution of amylose was of characteristic by botanical source as with that determined on a weight basis [19], but the difference in dp distribution with the source was visualized more clearly on a molar basis than on a weight basis. From the fluorescent response profiles except for rice and maize, the presence of two or three molecular species was obvious. The rice and maize amyloses gave a similar fluorescent profile showing a sharp (and apparently single) peak, but a faint shoulder was observed around the retention time of the peak of an RI response profile, suggesting also two (a major and another minor) molecular species. It was of interest that in the case of the rice and maize amyloses, molar-based distributions conflicted with weight-based distributions that implied three major molecular species. Distribution of all amyloses on a molar basis revealed that small molecules are a major species by number and occupy only a small proportion by weight. The above results on rice and maize agreed with those reported previously [8,9].

Because an accurate determination of the highest and the lowest dp values was difficult from the chromatograms, the range of apparent dp distribution (Table 1) was expressed conveniently as dp values of both the positions at which 10 and 90% of amylose molecules were eluted (Fig. 3). The tuber amyloses had about a 2–3 times wider distribution than the cereal amyloses. In the case of potato, barley, and maize, the elution position of 90% of molecules was out of the range of dp plots. But its dp value could be obtained from the extrapolated line because the ranges of dp plot for eight specimens overlapped with each other between 10^2 and 2×10^4. For detailed comparison, a fluorescence response profile was divided into subfractions by dp for each 10^3 and mol percentages of each subfraction were calculated (Fig. 4(A)). The cereal and tuber amyloses were quite different in dp distribution. The tuber amyloses showed a very wide dp distribution and contained a significant amount of very large molecules ($dp > 10^4$). On the other hand, all of the cereal amyloses showed a similar dp distribution with a predominance of molecules with a dp below 10^3. The subfraction with a dp below 10^3 was further divided by dp for each 10^2 (Fig. 4(B)). The cereal amyloses showed distributions characteristic to each source, and it was of interest that the maximum proportion appeared at dp $2–3\times10^2$ except for indica rice (dp $3–5\times10^2$). The dp distribution in japonica rice was similar to that in maize, but different from that in indica rice. The tuber amyloses showed distinct patterns from the cereal amyloses. The subfraction with a dp below 10^2 was not detected in any specimens. This appeared to be caused by the failure to form a 1-butanol complex during starch fractionation, rather than an absence of the corresponding amylose in starch, because linear, small molecules were found in the amylopectin fraction of amylomaize starch [21]. The results (Fig. 4) revealed that

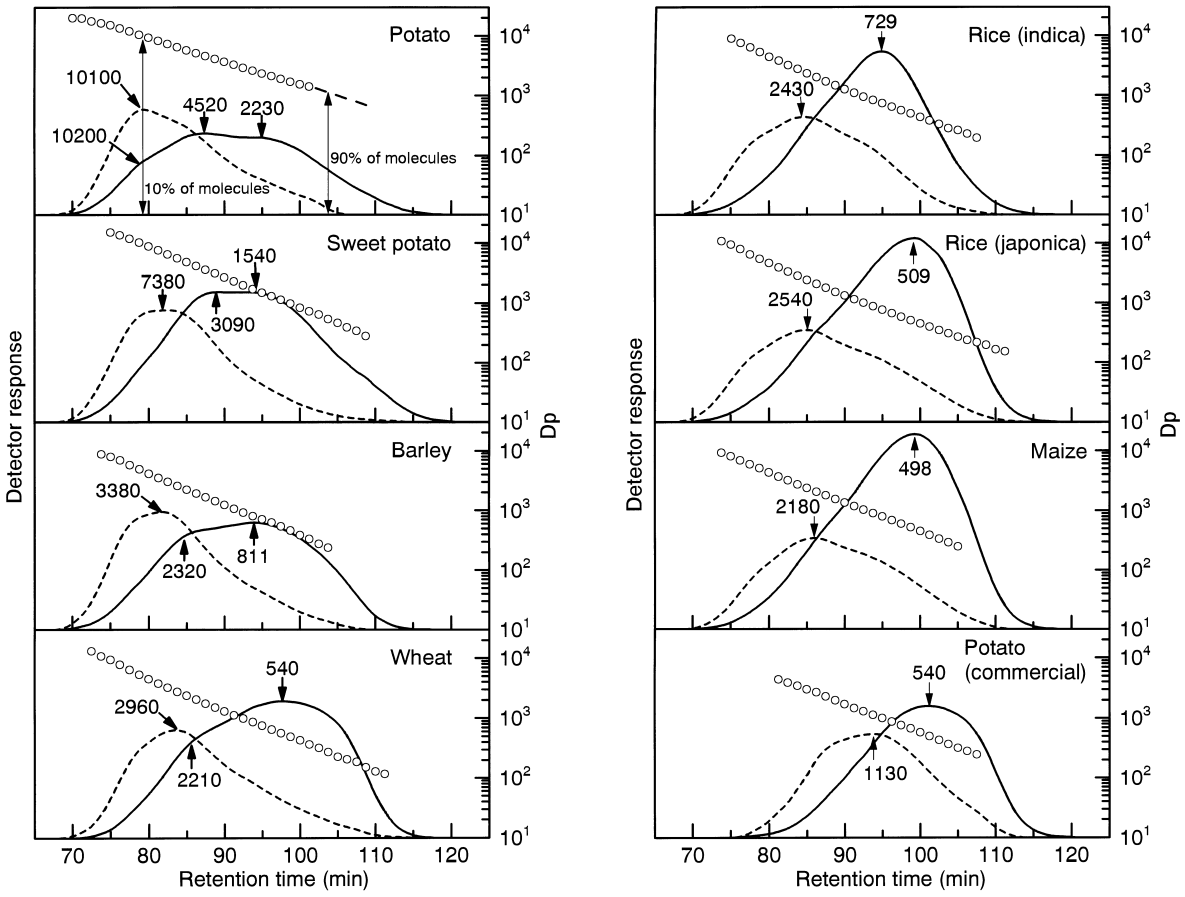

Fig. 3. Chromatograms of labeled amyloses: solid line, fluorescence response; dotted line, RI response; ○, dp. Numbers with arrows indicate dp. The dp was calculated from the RI/F value, of which each response was measured as the height at corresponding elution position, using the calibration line in Fig. 2.

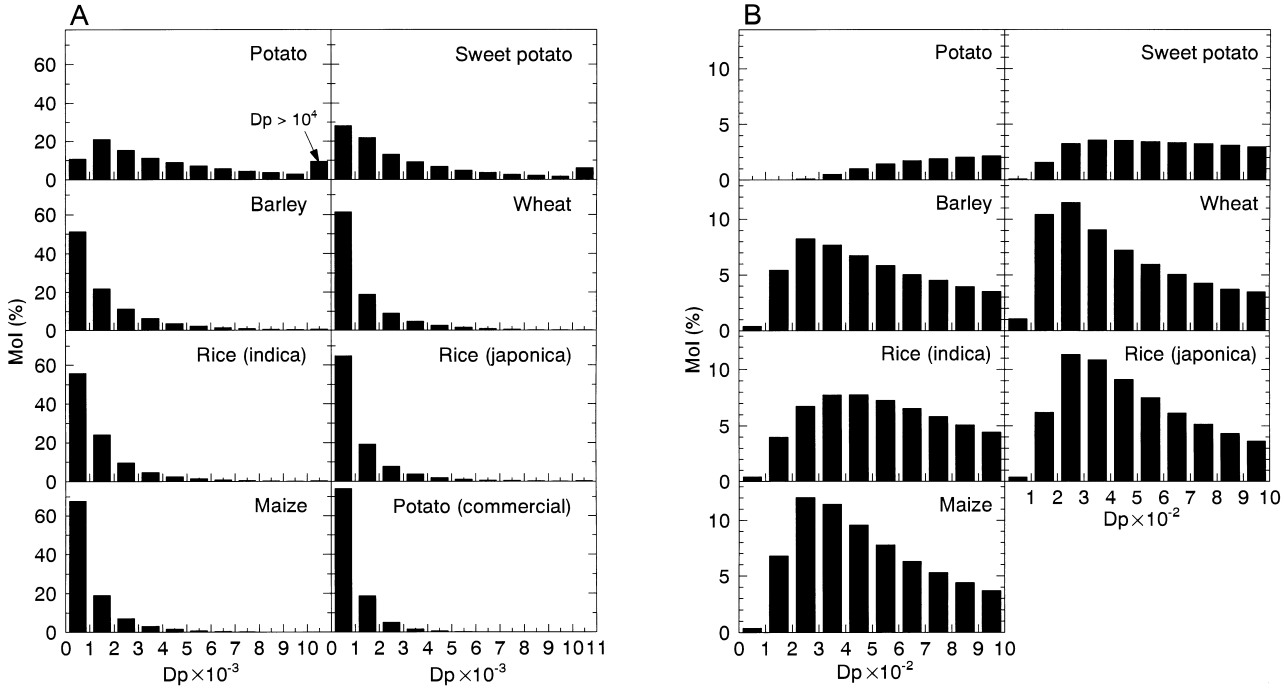

Fig. 4. Dp distribution expressed by mol percentage for each dp 10^3 (A) and dp 10^2 (B).

the major molecular species of amylose had a much lower dp than dp_n for whole amylose, and also suggested that the dp distribution of amylose is determined by different mechanisms during the biosynthetic process in tubers and cereals.

Granule-bound starch synthase (GBSS) is believed to play a role in amylose biosynthesis *in vivo*. GBSS has been found in a few isoforms. The difference in dp distribution of amylose might depend on the amount and enzymic properties of each isoform. Another possible explanation is that the amount of primers for amylose synthesis determines dp_n of amylose. However, it is difficult to examine these possibilities due to very limited knowledge on the role of each GBSS isoform and on primers.

The method described here will be applicable to amylose having a $dp > 10^2$ and probably many kinds of polysaccharides having reducing terminals if they are precipitated by solvent (e.g. ethanol). Examination of chain-length distribution of amylopectin is now under way.

References

[1] D.J. Manners, *Carbohydr. Polym.*, 11 (1989) 87–112.
[2] S. Hizukuri, *Starch: Analytical Aspects*, in A.-C. Eliasson (Ed.), *Carbohydrates in Food*, Marcel Dekker, New York, 1996, pp 347–429.
[3] K. Koizumi, Y. Kubota, T. Tanimoto, and Y. Okada, *J. Chromatogr.*, 464 (1989) 365–373.
[4] I. Hanashiro, J. Abe, and S. Hizukuri, *Carbohydr. Res.*, 283 (1996) 151–159.
[5] K. Koizumi, M. Fukuda, and S. Hizukuri, *J. Chromatogr.*, 585 (1991) 233–238.
[6] L.A. Larew and D.C. Johnson, *Anal. Chem.*, 60 (1988) 1867–1872.
[7] K.S. Wong and J. Jane, *J. Liq. Chromatogr. Relat. Technol.*, 20 (1997) 297–310.
[8] Y. Takeda, N. Maruta, and S. Hizukuri, *Carbohydr. Res.*, 227 (1992) 113–120.
[9] Y. Takeda, S. Tomooka, and S. Hizukuri, *Carbohydr. Res.*, 246 (1993) 267–272.
[10] M. Stefansson and M. Novotny, *Carbohydr. Res.*, 258 (1994) 1–9.
[11] M.G. O'Shea and M.K. Morell, *Electrophoresis*, 17 (1996) 681–688.
[12] P. Jackson, *Biochem. J.*, 270 (1990) 705–713.
[13] P. Jackson, *Methods Enzymol.*, 230 (1994) 250–265.
[14] S. Hase, T. Ikenaka, and Y. Matsushima, *Biochem. Biophys. Res. Commun.*, 85 (1978) 257–263.
[15] Y. Takeda, S. Hizukuri, and B.O. Juliano, *Carbohydr. Res.*, 148 (1986) 299–308.
[16] Y. Takeda, H.-P. Guan, and J. Preiss, *Carbohydr. Res.*, 240 (1993) 253–263.
[17] S. Hase, T. Ibuki, and T. Ikenaka, *J. Biochem.*, 95 (1984) 197–203.
[18] S. Hizukuri, Y. Takeda, M. Yasuda, and A. Suzuki, *Carbohydr. Res.*, 94 (1981) 205–213.
[19] S. Hizukuri and T. Takagi, *Carbohydr. Res.*, 134 (1984) 1–10.
[20] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350–356.
[21] C. Takeda, Y. Takeda, and S. Hizukuri, *Carbohydr. Res.*, 246 (1993) 273–281.