Carbohydrate Research, 84 (1980) 307–313
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

RANDOM SUBSTITUTION OF AMYLOSE

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(Received February 28th, 1980; accepted for publication, March 19th, 1980)

ABSTRACT

The production of modified amyloses often involves the *p*-toluenesulfonylation of the primary hydroxyl group of the D-glucopyranosyl residues, but it was not known whether this esterification is random when ~20% of the D-glucopyranosyl residues have been substituted. The reaction of *p*-toluenesulfonyl chloride with amylose in pyridine, in C-methylpyridines, in aqueous 2M KOH, and in dimethyl sulfoxide–pyridine yielded unsatisfactory products. However, *p*-toluenesulfonylation of 2,3-di-*O*-acetylamylose in pyridine yielded products that were converted into 2,3-di-*O*-acetyl-6-deoxy-6-iodoamylose, and thence into 6-deoxyamylose, a suitable substrate for the amylase to be used in the determination of random substitution. The hydrolysis products formed by the action of the crystalline, liquefying amylase of *Bacillus subtilis* were separated and analyzed. When a summation of the minimum number of separated, modified D-glucopyranosyl residues was compared to a computer-based calculation of the clustering expected, the results showed random esterification.

INTRODUCTION

There are many references in the literature to amyloses that have been chemically modified at either the primary hydroxyl group, or one of the secondary hydroxyl groups. Some of the earliest products were prepared by Whistler and co-workers[1] and by Bines and Whelan[2]. In many cases, these modified products were merely to be tested as substrates for carbohydrase systems. Other investigators prepared modified polysaccharides for other uses; thus, Wolfrom and Wang[3] and Horton and Just[4] used a series of modifications to prepare products that had some heparin-like activity, and Taylor[5] prepared some modified polysaccharides that seemed, in tests with chicks, to have more antihypercholesterolemic activity than the native polysaccharide.

Many of these products had only part of the D-glucopyranosyl residues modified, but there has always been uncertainty as to whether the modified D-glucopyranosyl residues in the polymer are randomly distributed, or are clustered in one part of the polymer.

Because many of the preparations have involved *p*-toluenesulfonylation of the primary hydroxyl group, it was decided to try to determine the extent of clustering

in this reaction. Whistler and Hirase[1] performed the most complete study of 6-*O*-tosylamylose. They conducted the esterification of both amylose and 2,3-di-*O*-acetylamylose with *p*-toluenesulfonyl chloride in pyridine. The same two starting compounds were used in the current study, but it was considered desirable to keep the degree of substitution (d.s.) at ~0.2, a level that has been found convenient in amylase studies.

It was important to have the amylose so modified that it would be hydrolyzed by alpha amylases, because the liquefying alpha amylase of *B. subtilis* was to be used to ascertain whether the chemically modified D-glucopyranosyl residues were randomly distributed. Previous studies with this enzyme had demonstrated that (*a*) no chemically modified monomer had ever been found in the hydrolyzate, (*b*) the enzyme cannot catalyze hydrolysis between two chemically modified D-glucopyranosyl residues, and (*c*) it is possible to identify those di- and tri-saccharide hydrolysis products that contain chemically modified monomers[6]. At d.s. values of ~0.2, a reasonable yield of these di- and tri-saccharides may be obtained; at higher d.s. levels, there is very little hydrolysis, and it is almost impossible to determine the structure of the highly clustered, high-molecular-weight oligosaccharides. The current investigation thus resembles previous ones conducted in this laboratory[6], but, in this case, it was necessary to have a complete materials-balance.

RESULTS AND DISCUSSION

1. Direct p-*toluenesulfonylation of amylose*

A. In nitrogen-base solvents. — The reaction in pyridine was conducted as described by Whistler and Hirase[1], with quenching as soon as the reaction mixture became homogeneous, in order to keep the d.s. values as low as possible.

Enzyme experiments conducted with products prepared by this direct method suggested that the reaction product was not homogeneous. Small samples were then suspended in boiling water for 30 min, and filtered, and the water-soluble and -insoluble portions were dried, and analyzed for content of sulfur. Based on the sulfur content, there was complete recovery, but, in all cases, the water-insoluble fraction contained a much higher proportion of sulfur than the water-soluble. Even extraction of a large sample with hot water in a Soxhlet apparatus showed a higher sulfur content in the water-insoluble fraction. It seemed probable that the small proportion of amylose that initially dissolved in the pyridine became extensively tosylated before the rest of the amylose had dissolved. This effect would be most noticeable at low d.s. values, where the reaction was quenched as soon as the mixture became homogeneous.

Experiments were also conducted in 2,6-dimethylpyridine, and in 2,4,6-trimethylpyridine, both of which are stronger bases than pyridine, but the reaction mixtures did not become homogeneous, and the sulfur analyses were very low.

Direct *p*-toluenesulfonylations were also conducted by adding pyridine and *p*-toluenesulfonyl chloride to a solution of amylose in dimethyl sulfoxide, but the

sulfur contents of the products were very low. When large proportions of pyridine and *p*-toluenesulfonyl chloride were used, over half of the product was lost on dialysis, and the lyophilized residue from the dialysis sacs was water-soluble, indicating that extensive degradation of the amylose polymer had occurred. It seemed possible that some of the primary hydroxyl groups (or tosylated primary hydroxyl groups) had been oxidized to the aldehyde, followed by cleavage by beta-elimination. Horton and co-workers[7] found similar degradation in their work with 6-aldehydoamylose. An alkaline solution of the current product showed the same, strong absorption-band, at 250 nm, as that of 6-aldehydoamylose[7].

B. In aqueous 2M potassium hydroxide. — It is also possible to conduct *p*-toluenesulfonylations in aqueous 2M potassium hydroxide, a medium in which amylose is soluble. When the products from this reaction were subjected to the action of sodium iodide in *N,N*-dimethylformamide, less than half of the tosylate groups were displaced, suggesting that much of the originated substitution had occurred at secondary hydroxyl groups.

2. p-*Toluenesulfonylation of 2,3-di-*O*-acetylamylose*

The procedure of Whistler and Hirase[1] was used. 2,3-Di-*O*-acetylamylose cannot, however, be used as a substrate for the enzyme, and the acetyl groups cannot be removed by the usual treatment with sodium methoxide in methanol without also removing the tosyl groups; indeed, Whistler and Hirase[1] used this method to prepare 3,6-anhydroamylose.

Ammonia in methanol has been used to remove the acetyl groups from sulfonic esters of partially acetylated monosaccharides without removing the tosyl groups[8]. The calculated and found values for the sulfur contents of three samples that had been deacetylated with ammonia in methanol showed that 45–55% of the *p*-toluenesulfonic ester groups had also been removed. Acid hydrolysis, followed by paper chromatography, confirmed the presence of 3,6-anhydro-D-glucopyranose. Thus, the procedure may be quite useful for monomeric compounds, because a separation can be effected, but this is not the case for the polymer, where the 3,6-anhydro-D-glucopyranosyl by-product is part of the same molecule.

Bines and Whelan[2] used $LiAlH_4$ to produce 6-deoxyamylose, a modified amylose that is sufficiently soluble to be used as a substrate for amylase. This reagent also removes the acetyl groups, but it may yield a product containing a considerable proportion of hydrated aluminum oxide as a contaminant. Sannella and Whistler[1] removed this contaminant in their 6-aminoamylose by dialysis in M hydrochloric acid. This is a good method for 6-aminoamylose, because the glycosidic bonds therein are less subject to acid hydrolysis than those of amylose[9], but it is not suitable for 6-deoxyamylose, in which the glycosidic bonds are more subject to acid-catalyzed hydrolysis than those of amylose[9]. We found that the oxide contaminant of the reduction products could be markedly lessened either by reacetylation, or by treatment with aqueous tartaric acid. Acid hydrolysis of the samples then yielded 6-

TABLE I

ACTION OF AMYLASE[a] ON 6-DEOXYAMYLOSE

	Product	*Product (μmol)*	*Total monomer (μmol)*	*Modified monomer (μmol)*
1	D-Glucose	33.4[b]	33.4	0
2	Maltose	52.5[b]	105	0
3	Maltotriose	36.0[b]	108	0
4	Maltotetraose	3.69[b]	14.76	0
5	6^2-Deoxymaltose	26.4	52.8	26.4
6	3,6-Anhydromaltose[c]	9.56	19.1	9.56
7	6-Deoxymaltotriose	16.8	50.4	16.8
8	Dideoxymaltotetraose	5.2	20.8	10.4
9	6-Deoxy-6-iodomaltotetraose	2.76	11.0	5.52
10	Bottom fraction			
	Glucose	25.3[d]	25.3	0
	6-Deoxyglucose	8.85[d]	8.85	8.85
Totals			450	77.5

[a]Crystalline, liquefying amylase of *Bacillus subtilis*. [b]Amounts determined by iodometric analyses of samples eluted from paper. [c]*O*-(3,6-Anhydro-α-D-glucopyranosyl)-(1→4)-D-glucopyranose. [d]Amounts determined by iodometric analyses after samples had been eluted, hydrolyzed (acid), separated by paper chromatography, and eluted.

deoxy-D-glucose and D-glucose, but, in all cases, the samples contained more 3,6-anhydro-D-glucose than was deemed permissible for use in this investigation.

Sodium borohydride has been reported to give almost quantitative yields on reduction of primary iodo compounds[10], and so the 2,3-di-*O*-acetyl-6-*O*-tosylamylose was converted into 2,3-di-*O*-acetyl-6-deoxy-6-iodoamylose, and this was treated with sodium borohydride in dimethyl sulfoxide, a reagent that also removes the acetyl groups.

Several samples of 6-deoxyamylose were prepared that had degrees of substitution ranging from 0.09 to 0.64. These d.s. values were based on the sulfur analyses of the 2,3-di-*O*-acetyl-6-*O*-tosylamyloses, and on analyses of the acid hydrolyzates of the final products. The latter analyses were conducted by gas–liquid chromatography of the trimethylsilyl ethers of the alditols, and by paper-chromatographic separation of the acid hydrolyzates followed by iodometric analysis.

A 6-deoxyamylose of d.s. 0.18 was chosen as the sample to be investigated for random substitution. It was subjected to the action of the crystalline, liquefying amylase of *Bacillus subtilis*, and then to extensive, paper chromatography[6]. The final assemblage of ten fractions is shown in Table I.

The identities of fractions 1, 2, 3, and 4 were confirmed by chromatography. The identities of fractions 5, 6, 7, and 8 were established by e.i.-mass spectrometry of the peracetates, and have been published[6]. Fraction 9 is listed in Table I as a 6-deoxy-6-iodomaltotetraose. It is probably larger than a maltotetraose, but the mass-

TABLE II

CLUSTERING OF MODIFIED UNITS IN RANDOMLY SUBSTITUTED POLYMER

Modified units present as groups of	*Percentages at increasing degrees of substitution (d.s.)*				
	0.1	*0.2*	*0.3*	*0.4*	*0.5*[a]
1	81.50	64.47	49.96	35.75	25.06
2	16.40	26.43	28.44	29.42	24.34
3	2.10	6.60	13.40	16.95	20.67
4		2.07	4.98	9.67	12.56
5		0.33	1.94	4.46	7.25
6			1.00	2.20	4.20
7			0.23	0.88	2.73
8				0.53	2.08
9				0.15	0.81
10					0.30

[a]The values for d.s. 0.5 were calculated from the results on 200 samples. The third batch of 100 contained two samples that consisted of a single cluster of 11 consecutive, modified units which were not counted, because the program was set to count only clusters of 2 through 10.

spectral datum for the molecular ion of the peracetate is missing. The usual ion, *m/e* 331, is present, as are the ions having *m/e* values 273 and 259, associated with the acetylated, 6-deoxyglycopyranosyl residue. There is a very large peak at *m/e* 127 (iodine), and one almost as large at *m/e* 141 (CH_2I^+). Although the presence of a peak at *m/e* 127 is not conclusive proof of the presence of a 6-deoxy-6-iodo-D-gluco-pyranosyl residue, the additional *m/e* 141 usually constitutes sufficient evidence. A small peak at *m/e* 399, and a slightly larger one at *m/e* 385, are correct values for a peracetylated 6-deoxy-6-iodo-D-glucopyranosyl residue minus one acetate and[11] minus (one acetate + 14), respectively.

Fraction 10, a mixture of the oligosaccharides, never moved far from the origin of the chromatographic paper. No attempt was made to isolate the pure components. The extract was concentrated, and the material hydrolyzed with mineral acid. The products, D-glucose and 6-deoxy-D-glucose, were separated by paper chromatography, and analyzed for reducing sugar[12].

The ratio of μmol of modified monomer to total μmol of monomer in Table I yields a d.s. of 0.17, in reasonably good agreement with the analysis of d.s. 0.18 for the sample used.

The modified D-glucopyranosyl residues in the compounds in fractions 5, 6, and 7 are definitely not in clusters of two (or more) adjacent, modified residues. Even if it is assumed that the modified residues in fractions 8, 9, and 10 are in clusters, it means that 52.8 (68.1%) of the total 77.5 of the modified D-glucopyranosyl residues are not clustered.

An interpolation of the data in Table II for d.s. 0.18 suggests that ~68% of the modified residues should be "isolated", or not clustered in units of two or more.

Thus, there is good evidence that the *p*-toluenesulfonylation of 2,3-di-*O*-acetylamylose is random.

EXPERIMENTAL

General. — Corn amylose (A. E. Staley) and crystalline *Bacillus subtilis* amylase (Enzyme Development Co.) were commercial samples. The reactions of *p*-toluenesulfonyl chloride with corn amylose in solution in dimethyl sulfoxide–pyridine were conducted for 48 h at room temperature. The final products were obtained by dialysis, followed by lyophilization. The reactions in 2M KOH were accomplished with *p*-toluenesulfonyl chloride (18 g) and corn amylose (20 g), in an ice–water bath, with precipitation by pouring the reaction mixture into an excess of ethanol. Deacetylation of 2,3-di-*O*-acetyl-6-*O*-tosylamylose by ammonia was accomplished by stirring the ester (10 g) in methanol (200 mL) presaturated with ammonia gas, for seven days, at which time the i.r. spectrum of a pellet of the product did not show any carbonyl peak. The preparation of 6-deoxyamylose, d.s. 0.18, has been described[6].

Methods. — The paper-chromatographic separation of fractions, and the identification of the fractions, have been described[6]. Iodometric analyses for reducing sugars were conducted by the method of McCleod and Robison[12]. A Perkin–Elmer Model 881 flame-ionization gas–liquid chromatograph equipped with a stainless-steel column (SE 32; 2.44 m) was used to determine the ratios of the trimethylsilyl ethers of the reduced monosaccharides prepared from the acid hydrolyzates of the 6-deoxyamyloses.

Enzymic hydrolyses. — These procedures have been described[6].

Computer program for random substitution. — POLY is a program that simulates the random substitution of groups on a polymer of high molecular weight. It was written for the IBM 370/158 batch-system.

The incidence of clusters in the polymer chain was the objective in the writing of POLY. A simple count of the clusters of 2 through 10 was requested. An array of length 100, representing the polymer, was initialized at zero, a random-number generator picked an address in the array, and a "one" was placed at that address. When the specified number of substitutions was obtained, the program counted the number of occurrences of the groups, and looped back to the starting point, setting the array at zero again. If a chosen address was already occupied by a "one", a new address was chosen, and the program was continued. The process was performed 300 times for each of the d.s. values shown in Table II.

A program called SCALER, which produces a seed value for the random-number generator, was used prior to POLY. SCALER tests the seed, to guarantee random numbers between 1 and 100.

POLY is successful in producing uniform results. The choice of a new address when the site chosen represents the actual conditions in the *p*-toluenesulfonylation of 2,3-di-*O*-acetylamylose might be the only problem with the computer program.

Fortunately, the necessity of making the second choice was not a serious problem at d.s. 0.1. At the higher d.s. values (>0.2), it would not have been possible to use the program without the second-choice option.

ACKNOWLEDGMENT

This work was supported by NIH Biomedical Research Grant RR 7059.

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