Carbohydrate Research, 83 (1980) 321–326
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

EVIDENCE OF MULTIPLE BRANCHING IN THE LEVAN ELABORATED BY *Streptococcus salivarius* STRAIN 51

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(Received October 31st, 1979, accepted for publication, November 20th, 1979)

ABSTRACT

Enzymic degradation of the levan elaborated by *Streptococcus salivarius* strain 51 followed by linkage analysis revealed that the levan possesses multiply-branched chains (B-chains) Large segments of the levan are arborescent

INTRODUCTION

There have been few reports concerned with structural details of the levans and little is known about the sequence of their (2→6)- and (2→1)-linked β-D-fructofuranosyl residues Speculation about the type-structure has been made[1], but, to our knowledge, no chemical evidence has been advanced The lack of suitable fructan hydrolases has probably hindered progress in this area (*cf* glucan analysis by enzymic methods[2,3]) Although a (2→6)-β-D-fructan 6-levanbiohydrolase has been reported[4] and an inulase has been induced[5], most known fructan hydrolases are probably β-D-fructofuranosidases possessing different degrees of substrate specificity No systematic study of their mode of action has been made[6] An extracellular β-D-fructofuranosidase[7], inductively produced by *Streptococcus salivarius* strain 51, hydrolyses the (2→6)- and (2→1)-β-D linkages in the levan of *S salivarius* strain 51 and the (2→1)-β-D linkage in inulobiose, but not the (2→1)-β-D linkages in inulin

The average repeating-unit of the levan[8] elaborated by *S salivarius* strain 51 contains ~9 β-D-fructofuranosyl residues The branching is through a single (2→1) linkage[7] (**1**) and the branches through this linkage contain several β-D-fructofuranosyl residues[8] The types of chains that can occur in this levan are therefore analogous to those in the three possible structures (comb-like, **2**, laminated, **3**, and arborescent, **4**) of a branched homopolysaccharide[9–11], and which have been distinguished[12] as A-, B-, and C-chains A comb-like segment possesses only A-chains in addition to the basal C-chain A laminated *segment* possesses only B-chains Intermediate proportions of A- and B-chains are present in arborescent segments We have used the exo-acting enzyme-preparation of *S. salivarius* strain 51 to investigate the types of chains that occur in this levan

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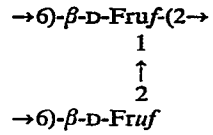

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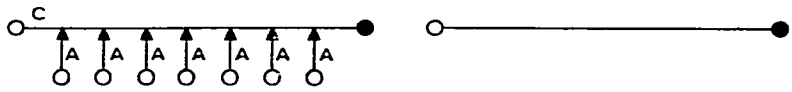

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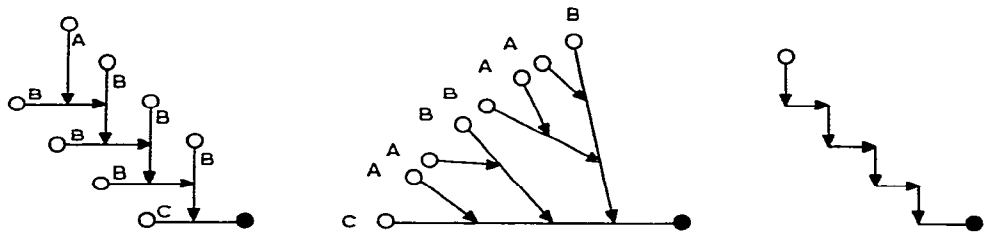

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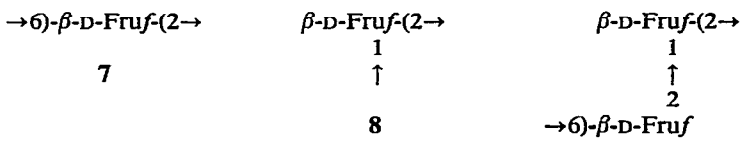

7 8 9

Fig 1 Structural units of levan and diagrammatic representations of its possible structures In **2–6** ○, non-reducing end-group. ●, reducing end-group, ———, chains of (2→6)-linked β-D-fructofuranosyl residues, arrow-heads, (2→1)-β-D-fructofuranosidic inter-chain linkages

RESULTS AND DISCUSSION

It was conceived that, during the course of the degradation by the exo-acting β-D-fructofuranosidase, the types and proportions of the glycosidic linkages in the residual polysaccharide could depend on the type-structure. The vicinity of *each* branching β-D-fructofuranosyl residue in structures **2** and **3** can be reached by the enzyme by step-wise hydrolyses of the consecutive (2→6) linkages of the A- and B-chains, respectively If the enzyme could then hydrolyse the linkages to C-1 (in **2**) and to C-6 (in **3**) of the branching β-D-fructofuranosyl residue, comb-like (**2**) and laminated segments (**3**) would essentially be debranched (*i e* , **2→5** and **3→6**) The removal of A-chains, which are attached by a (2→1) linkage to either the basal C-

chain (in **2** and **4**) or to B-chains (in **3** and **4**), would simply generate *additional* β-D-fructofuranosyl residues linked only through C-2 and C-6 (**7**) On the other hand, removal of *outer* sections of B-chains of **3** and **4**, which are attached to the branching β-D-fructofuranosyl residue by a (2→6) linkage, would generate β-D-fructofuranosyl residues linked only through C-2 and C-1 (**8**), a structural feature not present in the native levan A distinction between the comb-like segment (**2**) and segments possessing the multiply-branched B-chains might therefore be made by linkage analysis before and after partial enzymic degradation of the polysaccharide The pertinent aspect of this procedure is to ascertain the presence in, or absence from, the partially degraded polysaccharide of the structural unit **8**

The levan was therefore treated with the β-D-fructofuranosidase for up to ~3 5 h The degree of hydrolysis was determined from the amount of D-fructose released (Table I) Portions of each partially degraded polysaccharide were then subjected to the methylation–degradation procedure developed by Lindberg and his co-workers[13] By this procedure, structure **7** would give **10**, whereas structure **8** would give **11** These compounds are not easily distinguished by g l c and, furthermore, cannot be distinguished by electron-impact mass spectrometry (e i m s) The reduction step in the methylation–degradation procedure was therefore performed with sodium borodeuteride[8] By this method, segment **7** would give 2,5,6-tri-*O*-acetyl-1,3,4-tri-*O*-methylhexitol-*2-d* (**12**), whereas segment **8** would give 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylhexitol-*2-d* (**13**) These compounds can be readily distinguished by e i m s if analysed separately When in admixture, their detection is made difficult due to fragments containing ^{13}C [*i e* , $(m + 1)/z$], or fragments which are often of uncertain origin [*i e* , $(m - 1)/z$] It was also for this reason that the levan was hydrolysed to different extents, thus allowing comparisons of the various mass spectra

G l c of the hexitol-*2-d* derivatives obtained from each levan sample gave the components expected from the undegraded levan, *i e* , 2,5-di-*O*-acetyl-1,3,4,6-tetra-*O*-methyl-, 2,5,6-tri-*O*-acetyl-1,3,4-tri-*O*-methyl-, and 1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-

TABLE I

LINKAGE ANALYSIS OF NATIVE AND ENZYMICALLY DEGRADED LEVANS

Degree of hydrolysis of levan (%)	*Branching D-fructose residues (%)*	*Ratios of abundances of fragments in the mass spectrum of the tri-O-acetyl-tri-O-methylhexitol-2-d fraction*		
		Fragments (m/z) *161/162*	*190/189*	*205/206*
0	11 5	0 14	0 10	0 18
7 9	11 8	0 18	0 14	0 25
15 8	11 2	0 15	0 13	0 26
35 3	10 4	0 19	0 13	0 24
63 2	10 1	0 17	0 11	0 23

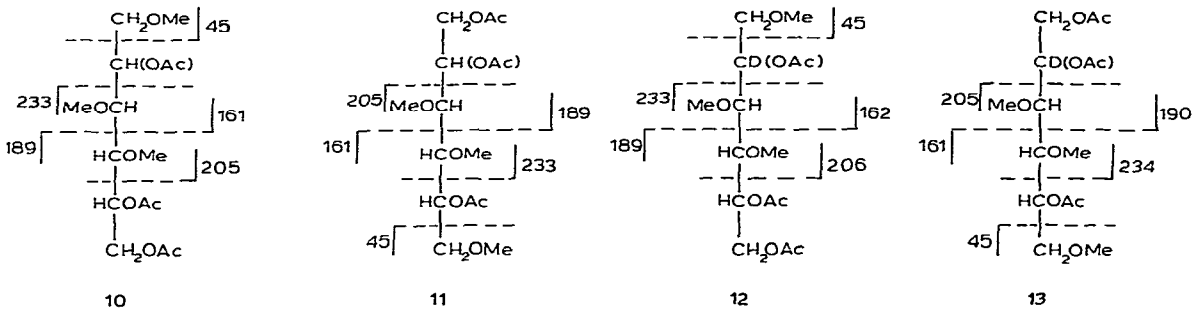

Fig 2 E I -induced fragmentation modes of tri-*O*-acetyl-tri-*O*-methylhexitol products (stereochemistry at C-2 not shown)

methylhexitol-*2-d* (see Table I of ref 8 for details) Table I shows the ratios of abundances of corresponding pairs of fragments which occurred in the mass spectra of the tri-*O*-acetyl-tri-*O*-methylhexitol-*2-d* fractions and which arise from structures **12** and **13** Such comparisons are valid, as the abundances of the various ions in the mass spectrum of most compounds are proportional to the pressure of their sample in the ion source[14] However, the values in Table I were obtained by direct measurements of peak heights and may thus be subject to small errors On the other hand, the values show a consistent increase in the relative abundances of the ions having *m/z* 161, 190, and 205 Therefore, the tri-*O*-acetyl-tri-*O*-methylhexitol-*2-d* fraction contained compounds **12** and **13** The percentage of branching β-D-fructofuranosyl residues in the native and partially degraded levan samples was determined[8] from the molecular proportions of the tri-*O*-methyl- to di-*O*-methyl-hexitol derivatives Re-methylation of portions of the methylated levans did not change these proportions Therefore, complete methylation had been achieved in all cases This finding shows that the formation of compound **13** was not the result of incomplete methylation, but that it indeed arose as a consequence of enzymic degradation of the levans We therefore conclude that the enzymically degraded levan possessed β-D-fructofuranosyl residues linked only through C-1 and C-2 (**8**); *i e* , the process **1**→**9** had occurred Consequently, segments of the native levan possessed B-chains (**3**) We believe these results to be the first chemical evidence of multiple branching in a levan

Table I shows that the percentage of branching β-D-fructofuranosyl residues remained essentially constant during the course of the degradation by the exo-acting enzyme This finding, together with the fact that outer sections of B-chains are removed at the same time (*i e* , **1**→**9**), shows that large segments of the native levan are arborescent (**4**) Such a structure is consistent with the spherical or ellipsoidal shape of other microbial levan molecules[15] and the mechanism of their enzymic synthesis[16], as well as the origin of the branches[1]

Kinetic data[17] on the acid hydrolysis of the levan of *S salivarius* strain ATCC 13419 suggest an initial, rapid reaction in competition with a slower reaction It was argued that the species present ($\overline{M}_r \sim 3 \times 10^6$–$6 \times 10^6$) at the time when the second

reaction begins to dominate would consist mainly of the skeletal backbone with little branching The assumed structure of that levan is different from that proposed here for the levan of strain 51 It is also at variance with the observation[8] that partial hydrolysis with acid of the levan of strain 51 yields, in addition to levanbiose and its homologues, a considerable amount of inulobiose together with appreciable quantities of oligosaccharides possessing intact branching-units, thereby demonstrating that the susceptibilities to acid hydrolysis of the (2→1)- and (2→6)-β-D-fructosidic linkages are comparable

Acid hydrolysates of the levan of *S salivarius* strain ATCC 13419, which had $\overline{M}_r$ between $\sim 2 \times 10^4$ and $\sim 8 \times 10^6$, have also been examined[18] for their physical properties and by ^{13}C-n m r spectroscopy The conclusion that the observed differences in physical properties either are not due to the average frequency of branching or are attributable to subtle differences in branching, which cannot be detected by ^{13}C-n m r spectroscopy, supports our finding on the susceptibilities to acid hydrolysis of the D-fructosidic linkages in levan The structure proposed for large segments of the levan of *S salivarius* strain 51 is also in agreement with the above observations

EXPERIMENTAL

A digest containing levan[8] [225 mg, in water (15 cm^3)] and *S salivarius* β-D-fructofuranosidase[7] (~4 5 units) in phosphate buffer (0 1M, pH 6 6, 67 5 cm^3) was incubated under toluene at 37° Samples were withdrawn and, after deactivation of the enzyme by immersion in boiling water, their reducing-saccharide content (as D-fructose) was determined by the Nelson method[19] The remainder of each sample was exhaustively dialysed against deionised water and freeze-dried The methylation of each levan sample and the characterisation of the *O*-acetyl-*O*-methylhexitols obtained from the methylated levans were performed essentially as described before[8]

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