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**DIMENSIONS OF THE SUBSTRATE SITE INVOLVED IN THE
ENZYMOLYSIS OF A POLYSACCHARIDE**

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N.R.C. No. 7405

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The specificity of an enzyme that degrades a polysaccharide is correlated generally with a particular kind of glycosyl unit or linkage in the substrate. However, little is known as to what portion of the polysaccharide molecule is involved in the reaction; whether a large region about the site of hydrolysis is affected, or only the immediate vicinity of the bond cleaved. Some information has now been obtained about spatial requirements in one specific instance, i.e., for the action of a "xylanase" on an arabino-xylan.

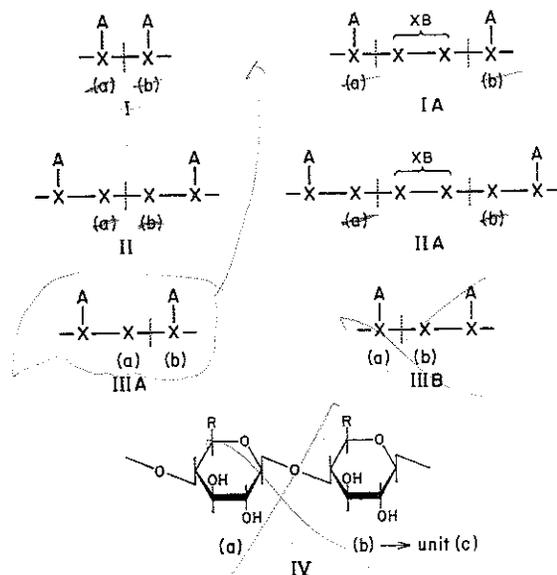
The polysaccharide examined is a soluble constituent of wheat flour (1-4). It contains a chain of β -D-xylopyranosyl units joined by (1 \rightarrow 4) bonds, and about two in five of these units serve as points of attachment also (through positions 2 and (or) 3) for L-arabinofuranosyl branches. An enzyme produced by *Streptomyces* sp. QM B814 (5) partially degrades this pentosan yielding

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several oligosaccharides, principally xylobiose and xylotriose, but liberating only traces of arabinose and xylose (6, 7). About 60% of the product consists of polymeric fragments having an average molecular weight (estimated to be about 4000 for the derived acetate; also see below) less than 1/10 that of the original polymer (1). Hence most of the arabino-xylan molecules must be degraded at least in part by the enzyme.

Formation of the polymeric fragments involves the exposure of new end-units, which may or may not contain arabinosyl branches depending on how



close to branch-points the attack takes place. Thus reducing (a) and non-reducing (b) end-units of fragments generated from I or IA (in which the formation of xylobiose (XB) is also represented) possess branches, whereas II or IIA affords a product in which (a) and (b) are unbranched. Periodate oxidation should permit ready differentiation between these alternatives since products of the latter type should liberate 2 moles of formic acid per molecule, as do oligosaccharides of the (1 → 4)-linked xylobiose series (8),* whereas little or no acid can be produced with branches present. Polymeric fragments derived from an intermediate type of structure (III, A or B) should yield one-half of the acid obtainable when both end-units are unbranched. Accordingly, four fractions of the polymeric fragments (obtained by gradient precipitation from aqueous solution with ethanol) were treated with excess sodium periodate at 12° C. Each fraction yielded formic acid and, by reference to the acid produced

*Under the oxidation conditions used 2 moles of acid per molecule are formed rapidly (ref. 8 and Experimental section); on prolonged oxidation, however, an additional mole of acid is released (8).

from xylotriose as a model compound, values for relative degrees of polymerization (D.P.) were found to be 30, 23, 17, and 15, respectively. Corresponding D.P. values for these fractions as estimated from copper-reducing equivalents (9) were 28, 22, 15, and 12, respectively. With such close agreement between the two sets of measurements it is clear that the reducing and non-reducing end-units of the polymeric fragments possess few, if any, arabinosyl branches.

The current data show that the *Streptomyces* enzyme cannot readily hydrolyze bonds joining branched xylosyl units of the flour pentosans (I). Rather, the minimal spatial requirement between branches appears to be two adjacent xylosyl units (II). Similarly, the formation of xylobiose, e.g., may be taken to involve a sequence of at least four consecutive unbranched units (IIA). These findings therefore complement other evidence (6, 7) showing that a large part of the pentosan molecule consists of highly-branched regions (as in III) in which the number of unbranched xylosyl units between branched units rarely exceeds one.

Earlier it was pointed out (7, 10, 11) that the specificity of some polyxylo-sidases may be related to certain structural features of the glycosyl unit (that is liberated as a reducing-unit on hydrolysis) rather than to the nature of the linkage cleaved. Similarly, the specificity of the *Streptomyces* xylanase may be described in terms of a 4-substituted β -D-xylosyl unit (*a* in IV, R = H) more adequately than in terms of a β -(1 \rightarrow 4) bond, since the arabinosyl branches do not alter the mode of linkage between the main-chain units but do modify the units themselves. However, the glycosidic center of unit *b* also is a 4-substituted β -D-xylosyl unit, but is not attacked. The cellulase produced by the same organism shows a similar differentiation in its attack on cereal β -glucans (11) and lichenin (12): i.e., in structure IV (R = CH₂OH) the 4-substituted β -D-glucosyl unit *a* is liberated but not unit *b*. Conceivably, cleavage of the bond linking *a* to *b* in IV involves binding sites in both units, e.g. the 3-hydroxyl groups, and the presence of a substituent at this position of unit *c* could block attack on the glycosidic center of *b*. The action of other xylanases on arabinoxylans (13, 14) and uronosyl-xylans (15) structurally related to the wheat flour pentosans can also be described in terms of the release of 4-substituted β -D-xylosyl units. However, these latter enzymes appear able to approach more closely to branch-points than does the *Streptomyces* enzyme since they yield oligosaccharides in which L-arabinose or 4-O-methyl-D-glucuronic acid is attached to the non-reducing end of a xylobiose or xylotriose unit.

Experimental

Isolation of Polymeric Fragments

Wheat flour pentosan (1.0 g, purified via the derived acetate (1)) in water (100 ml) was incubated at 45° C for 18 hours with an enzyme preparation (150 mg) from *Streptomyces* sp. QM B814. (Preliminary experiments show that the maximum degree of degradation of the polysaccharide is reached under these conditions (6).) The digest was then heated on the steam bath for 30

minutes, and concentrated at 45° C to a volume of 20 ml. Ethanol was added to the concentrate slowly with stirring; in 50% ethanol a dense brown precipitate separated out (this appeared to be mainly denatured enzyme), and at alcohol concentrations of 65% and 80%, respectively, two colorless flocculent materials were obtained. The latter products (A, 270 mg and B, 340 mg) were recovered by lyophilization.

Acetylation of A and B (100-mg samples) in formamide with acetic anhydride-pyridine under conditions given earlier (1), afforded colorless products readily soluble in acetone. Vapor-pressure thermometric measurements (16) on solutions of A- and B-acetates in acetone (concn., 8–9%) gave molecular weight values of 5100 and 2700, respectively. Viscometric measurements on the derived acetate of the original pentosan indicate that the molecular weight is in the vicinity of 70,000 (1).

End-group Analysis of Polymeric Fragments

Periodate oxidation.—Samples (50 mg) of A and B and of two comparable materials isolated in another experiment (6) were dissolved in water (each in 7 ml). Sodium metaperiodate solution (150 mg, 10 ml) was added to the solution of each fraction, and the reaction mixtures were stored in the dark at 12° C. A sample of crystalline xylotriase (10 mg) was oxidized simultaneously under the same conditions.

Portions of the reaction mixtures were analyzed for acid production at 18, 24, and 46 hours, by adding excess ethylene glycol, then potassium iodide, and titrating released iodine with standard thiosulphate. The yield of formic acid from xylotriase at 18 hours was 1.93 moles/mole, remaining essentially constant during the succeeding 28 hours reaction time (cf. (8)). Acid production from the polymeric fragments was also essentially constant during the period 18–46 hours. D.P. values for the latter materials were calculated, therefore, from the observed acid yield at 18 hours, relative to that of xylotriase at 18 hours, e.g.,

$$\begin{aligned} \text{D.P. fraction A} &= \frac{(\text{equiv. acid/g xylotriase}) \times (\text{base M.W.}) \times (\text{D.P. xylotriase})}{(\text{equiv. acid/g fraction A}) \times (\text{base M.W.})} \\ &= \frac{4.66 \times 138 \times 3}{0.484 \times 132} = 30.1. \end{aligned}$$

D.P. values based on reducing equivalent were estimated colorimetrically by the Somogyi copper-reducing modification (9), using crystalline xylotriase as standard.

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