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THE ATTACK MECHANISM OF AN EXO-1,3-β-GLUCOSIDASE FROM *BASIDIOMYCETE* SP. QM 806

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Summary

The attack mechanism of a purified exo-1,3-β-glucosidase (1,3-β-D-glucan glucohydrolase, EC 3.2.1.58) was investigated by using as a substrate a mixture of two structurally characterized periodate-oxidized and reduced unbranched 1,3-β-D-glucans which differed only at the reducing terminal. The substrates, derivatives of laminarin, were altered only at the terminals due to resistance of the internal (1 → 3)-linked glucosyl residues to periodate oxidation. Each glucan has only a single and identical altered non-reducing terminal per molecule.

Upon enzymatic hydrolysis, one molar equivalent of glycerol was produced from the altered non-reducing terminal of each substrate molecule attacked. Using glycerol as an indication of the number of chains acted upon, the quantity of D-glucose produced from the internal residues was used to determine the extent to which a chain was initially attacked.

The glucose to glycerol ratio during the course of the hydrolysis indicates that the enzyme proceeds by a multiple-attack mechanism where four glucosyl residues are successively removed per encounter from the non-reducing terminal of each substrate molecule.

Introduction

Exo-carbohydrases hydrolyze by either a multi-chain or single-chain mechanism of attack. In multi-chain attack the enzyme attaches to a non-reducing terminal chain of the substrate producing a single cleavage; the next cleavage occurs with attachment to a different substrate molecule or a different portion of the same molecule. In single-chain attack, the enzyme cleaves successive linkages of the same chain [1]. Examples of both types have been described. The transglycosylase from *Aspergillus niger* (α-D-glucoside glucohydrolase, EC 3.2.1.20) which attacks starch at the same rate as maltose, appears to proceed by a multi-chain mechanism [2], as do barley malt α-glucosidase (α-D-glucoside

glucohydrolase, EC 3.2.1.20) which also attacks starch [3] and plant and muscle phosphorylase (1,4-α-D-glucan: orthophosphate α-glucosyltransferase, EC 2.4.1.1) [4—6]; whereas *A. niger* glucoamylase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3) and β-amylase (1,4-α-D-glucan maltohydrolase, EC 3.2.1.2) appear to have a single-chain mechanism [7—9].

In the case of β-amylase, it has been suggested that the enzyme removes on the average four successive maltosyl residues per encounter [9]. This type of limited single-chain attack, termed multiple attack [9,10], is thus intermediate between a multi-chain and a complete single-chain mechanism [1].

The exo-1,3-β-glucosidase from *Basidiomycete* sp. QM806 (1,3-β-D-glucan glucohydrolase, EC 3.2.1.58) liberates single glycosyl residues from the non-reducing terminal of 1,3-β-D-glucans such as laminarin [11]. The action pattern, specificity, hydrolytic mechanism, kinetic parameters and physical properties of the purified enzyme have been described [11—14].

It has recently been reported that the exo-1,3-β-D-glucosidase also proceeds by a single-chain attack [17]. The present results suggest it is a multiple-attack type of single-chain mechanism.

Experimental

Enzyme preparation and analysis of activity. The purification of the enzyme and the procedure for assay have been described previously [11,12]. Enzymatic activity was determined by the Nelson—Somogyi method [18] for reducing sugars, or by a glucose oxidase method [19] modified to accommodate purified preparations of glucose oxidase free of contaminating carbohydrase activity. One unit of enzyme is that amount which will liberate 1 μmole of glucose or its reducing equivalent per minute under the prescribed conditions [11,12]. The enzyme preparation used in these experiments was highly purified and free from endo-1,3-β-D-glucosidase and other contaminating carbohydrase activities [11—13]. Its properties were the same as described previously [12,18].

Other analytical procedures. Total carbohydrate was determined by the phenol—H_2SO_4 method [20]. The usual method of glycerol determination [21,22] was modified [11] so that only free glycerol was determined. Under these conditions, free glucose does not interfere and the altered non-reducing terminal of the periodate-oxidized and reduced laminarin [11] is not affected. The contribution made by free glycoaldehyde was corrected for. The bound glycoaldehyde released in the final step of the determination makes a negligible contribution to the color development [11]. Glycerol standards were employed at the experimental concentrations described as controls to assure accuracy of the method.

Substrates. The source of the laminarin* used in this experiment was a preparation from *Laminaria hyperborea* (*Laminaria cloustoni*) [24], obtained from the Liverpool Borax Co., St. Paul's Square, Liverpool 3, England. The

* The term laminarin is used throughout. It is used here to designate *L. hyperborea* laminarin. Although the term laminaran, rather than laminarin, is in accordance with current nomenclature, it has not yet been officially accepted by the I.U.B.

initially cold water-insoluble laminarin* was fractionated into both soluble and insoluble components** which were characterized as described elsewhere [25].

In the present experiment, only the purified insoluble fraction was used. These components are unbranched 1,3-β-D-glucans which vary only at the reducing terminal. They consist of a linear component (insoluble laminarose) having a free reducing terminal and a similar 1-substituted mannitol terminal component (insoluble laminaritol) [25]. They were periodate oxidized and reduced with $NaBH_4$ [25] via the method of Smith and Montgomery [26]; borate was removed as described previously [11]. The ability of the purified exo-1,3-β-D-glucosidase to degrade periodate-oxidized and reduced laminarin derivatives has been demonstrated previously [11,13,25]. Under these conditions, as well as those described in the present experiments for enzymatic hydrolysis (0.05 M acetate buffer, pH 4.80, 37°C), the enzyme displayed the same relative rates on the substrates described [12,13,25]. There was no detectable non-enzymatic hydrolysis of the periodate-oxidized and reduced derivative.

Other materials. Purified glucose oxidase and horseradish peroxidase were obtained from Boehringer-Mannheim Corp., New York, N.Y. Purified *o*-dianisidine was obtained from the Sigma Chemical Co., St. Louis, Mo. All other materials were obtained as described previously [13].

Results and Discussion

The exo-glucosidase acts on periodate-oxidized and reduced laminarin at a rate 1/100 that observed on unmodified laminarin [11,13,25]. Enzymatic degradation of the laminarin derivatives yields glycerol and glycoaldehyde from the non-reducing terminal, and D-glucose from the internal residues that are inert to periodate [11]. If the derivative is subjected to dilute acid hydrolysis, 0.1 M HCl, for approx. 18 h at room temperature, rather than enzymatic attack, glycerol and glycoaldehyde are also released and the product is again susceptible to enzymatic attack at a rate similar to the unoxidized polysaccharide [11]. This is illustrated in Fig. 1.

In previous experiments [11,13] the laminarin, although postulated to have branched components, was not structurally characterized; thus the molar equivalent of glycerol released per polysaccharide molecule could not be determined since the number of non-reducing terminals per molecule was not

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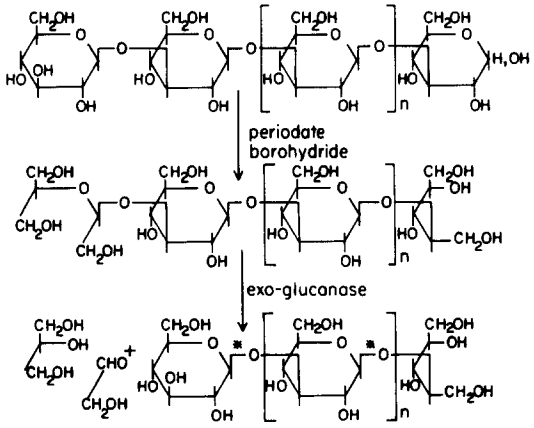
* The term insoluble laminarin has historically been used [23] to distinguish *L. hyperborea* (*L. cloustoni*) from other species of laminarin which are soluble in cold water (approx. 20°C). Insoluble laminarin consists of a mixture of reducing (G-chains) and non-reducing (M-chains) glucan components [23]. The reducing components here are designated laminarose; the non-reducing components are designated laminaritol.

** The terms soluble and insoluble are used here to refer to the solubility properties of the fractionated components of *L. hyperborea* laminarin upon standing at 20°C (cf. ref. 25). At the temperature and substrate concentrations used in the present experiments (37°C) the fractionated insoluble components were soluble and remained in solution throughout the course of the experiments. The insoluble components at 0.1% concentration eventually retrograde (precipitate) upon standing at room temperature (about several hours, 25°C), whereas the soluble components do not.



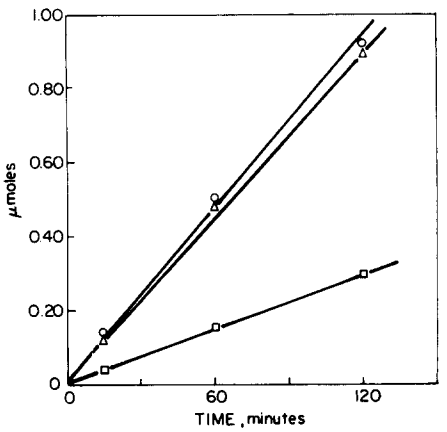


Fig. 1. Structural representation of dilute acid hydrolysis and exo-1,3-β-D-glucosidase hydrolysis of periodate-oxidized and reduced laminarin. The structure illustrated corresponds to the insoluble reducing component of laminarin as described in the text. Hydrolysis of the non-reducing terminal linkage by either enzyme or dilute acid results in spontaneous decomposition of the terminal residue to form glycerol and glycollic aldehyde. The asterisk (*) indicates the additional cleavage points during the enzymatic hydrolysis. Periodate oxidation under the conditions described in the text generates a stable formate ester residue at the anomeric carbon of the reducing terminal residue. This is subsequently hydrolyzed by the alkaline borohydride treatment to yield the arabitol residue shown.

Fig. 2. The action of the exo-laminarinase on the periodate oxidized and reduced insoluble components of laminarin. The values shown represent the difference between determinations made in the presence and absence of enzyme. The values in the absence of enzyme were negligible throughout. ▲, release of glucose as determined by the glucose oxidation procedure; ●, release of reducing equivalent to glucose as determined by the Nelson—Somogyi procedure; ■, release of glycerol. The incubation mixture consisted of 0.12 units of enzyme/ml, 0.10% substrate, and 0.05% gelatin in 0.05 M acetate buffer, pH 4.8. The reaction was conducted at 37°C and initiated by addition of substrate. Aliquots were removed at the intervals shown and placed in a boiling water bath for 3 min to denature the enzyme and were assayed for glucose, reducing capacity and glycerol as described in the text. These analyses were conducted on portions of the same aliquot.

known. In this experiment the fractionated and structurally characterized unbranched components of purified laminarin were used as a substrate. These components of *L. hyperborea* laminarin consist of approximately equal parts of a reducing 1,3-β-D-glucan component (insoluble laminarose) and a non-reducing 1,3-β-D-glucan component (insoluble laminaritol), the latter differing only in that it has a 1-substituted mannitol residue at the reducing terminal [25]. Both of these linear (unbranched) components have an average degree of polymerization of approx. 20—25. The components were periodate oxidized and reduced. Since disruption of the non-reducing terminal linkage by the enzyme can, under these circumstances (cf. Fig. 1), produce only one molar equivalent of glycerol per molecule, glycerol was used as a marker of the number of chains attacked and the quantitative relationship between the amount of glycerol and glucose released was determined. The results are shown in Fig. 2. Under the conditions employed for enzymatic hydrolysis there was no detectable non-enzymatic degradation of the altered non-reducing terminal [25].

The course of enzymatic hydrolysis of the altered substrates was followed during the initial phase of hydrolysis. The degree of hydrolysis at 120 min, assuming an average degree of polymerization of 20—25, represents about 15%

of the glucosyl residues present as estimated by the phenol—H_2SO_4 method on the altered substrates [20]. The rate of release of glucose and glycerol was constant throughout the interval measured. The mole ratio of glucose to glycerol was 2.9 at 15 min, 3.1 at 60 min, and 3.0 at 120 min. After 120 min, the ratio of glucose to glycerol gradually increased.

The relationship between the glycerol released and the glucose produced during the initial phase of hydrolysis was used as a means of distinguishing between the possible types of attack.

In the case of multi-chain attack, where only one cleavage occurs per substrate encounter, there would be an initial release of glycerol with essentially no apparent release of glucose until a sufficient quantity of chains with unmodified terminals had been generated for detectable enzymatic action. The enzymatically generated chains with intact non-reducing terminals should then produce a pronounced increase in glucose production due to the relative rates of hydrolysis on the two substrates [11,13]. This assumption is based on the finding that the enzyme shows approximately the same affinity for the laminarin derivative and the unmodified substrate on the basis of competitive inhibition [13]. It also implies that once a derivative binds to the enzyme, the enzyme—substrate complex proceeds in the same manner towards hydrolysis, even though at a slower rate, as does a molecule which has an intact non-reducing terminal. Thus, in the case of multi-chain attack, although the ratio of glucose to glycerol would initially be less than one, it would rapidly become greater than one as hydrolysis progressed, and would constantly increase in favor of glucose production. As can be seen (Fig. 2), this is not the case. The ratio is constant, and greater than one, indicating that from the onset less glycerol is produced than glucose. The constant ratio of glucose to glycerol observed in conjunction with the large difference in rate of 1 to 100 between the altered derivative and the substrate with an intact, non-reducing terminal indicates that a single, reversible, enzyme—substrate complex (a multi-chain attack) is not occurring. Even if a large difference in apparent K_m did exist between the altered and unaltered substrate, the substrate with an intact non-reducing terminal would be preferentially hydrolyzed. The fact that this does not appear to be the case kinetically indicates a type of irreversible enzyme—substrate binding process (a continued enzyme—substrate encounter) or, in effect single-chain attack; i.e. sequential attack on the same substrate molecule [1]. This argues against a multi-chain attack and suggests that the exo-glucosidase proceeds by some type of single-chain mechanism.

The extent of single-chain attack can be determined from the ratio of glycerol to glucose produced. If complete single-chain attack had occurred, there would be essentially total hydrolysis of the substrate molecule from the non-reducing terminal to some point near the reducing terminal. In such a case, although the ratio of glucose to glycerol would remain constant throughout hydrolysis, as was observed, the ratio would be approx. 20 to 1 (assuming an average number of 20—25 glucosyl residues per chain [25]). From the results, this was not the case; the ratio was approx. 3 to 1.

This indicates that the enzyme acts only to a limited extent on each chain at any one time. The ratio of 3 to 1 suggests that the enzyme removes on the average only four residues per encounter with a substrate molecule (the altered

terminal plus three glucosyl residues). This suggests that the exo-glucosidase proceeds by a partial or multiple-attack type of single-chain mechanism.

In the initial stages of hydrolysis of laminarioctaose by the exo-glucosidase, the corresponding lower homologs laminariheptaose, laminarihexaose and laminaripentaose were scarcely evident whereas laminaritetraose appears as a predominant product along with small amounts of laminaritriose (paper chromatography) [27]. This qualitative observation of a multiple-attack pattern on an oligosaccharide which resembles the linear (insoluble, unbranched) components of laminarin, is consistent with the present results. A similar observation has been reported using a borohydride-reduced *L. hyperborea* laminarin preparation [17]. In the case of lower homologs such as laminariheptaose and laminarihexaose, the pattern appears to revert towards a multi-chain type of attack; i.e. the next lower homolog becomes a more prominent product [27]. This indicates an altered mode of attack on substrates of less than optimal size for unimpeded multiple attack. A similar result has been noted in the case of β-amylase [28].

It has been observed that exo-glucosidases that invert configuration in the degradative direction do not catalyze transglycosylation reactions, whereas those that retain configuration frequently do [29,30]. The present reults suggest that these properties are also associated with the type of attack. Exo-carbohydrases that invert configuration appear to have a single-chain attack [7—9,17], whereas those that retain have a multi-chain attack [2—6]*. The current results substantiate this correlation between inversion of configuration and single-chain attack.

The multiple-attack type of single-chain mechanism has been convincingly demonstrated for β-amylase, although evidence for the number of residues removed per encounter is statistical due to the inherent structural uncertainty in preparation of the substrates [9,10,34]. The present results, using structurally characterized substrates, provide a further and more direct chemical substantiation of the multiple-attack mechanism.

The glucose to glycerol ratio produced by exo-1,3-β-D-glucosidase attack on the periodate-oxidized and reduced linear 1,3-β-D-glucans indicates that the enzyme is proceeding from the non-reducing terminal by a multiple-attack type of single-chain mechanism. The average number of successive residues removed is four, three glucosyl residues plus the altered terminal.

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* The interrelationship between the ability to catalyze transglucosylation and the type of attack mechanism holds only in the degradative direction with exo-carbohydrases. It does not appear to apply to synthetic or condensation reactions catalyzed by exo-carbohydrases, endo-carbohydrases [31], or disaccharide phosphorylases [29,32,33], except in the case of the polysaccharide phosphorylases [4—6].

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