THE MECHANISM OF CARBOHYDRASE ACTION

11. Pullulanase, an Enzyme Specific for the Hydrolysis of Alpha-1→6-Bonds in Amylaceous Oligo- and Polysaccharides[1,2]

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ABSTRACT

Pullulanase, an extracellular enzyme of *Aerobacter aerogenes*, is specific for the hydrolysis of alpha-1→6-glucosidic linkages in branched amylaceous polysaccharides and derived oligosaccharides. Its action and specificity are closely similar to the plant R-enzyme complex, but pullulanase has the additional capacity to effect a limited degree of hydrolysis of the branch linkages of glycogen, which R-enzyme, for steric reasons, does not. Steric hindrance to pullulanase action on both amylopectin and glycogen does also occur, but is lessened if the outer chains of the polysaccharide are first degraded by beta-amylase. The specificity of pullulanase has been defined by means of exposure to oligosaccharides of known constitution. It does not hydrolyze isomaltose, and there is an absolute requirement for alpha-1→4-glucosidic bonds in its substrates. It has no detectable action on compounds in which alpha-glucose is joined through a 1→6-bond to a maltosaccharide, while its action on alpha-maltosaccharides joined 1→6 to glucose is either negligible or so slow that it could be due to an enzymatic impurity. Pullulanase acts rapidly on alpha-1→6-bonds only when each of the components joined through the linkage is a maltosaccharide. Thus the linear tetrasaccharide 6^2-alpha-maltosylmaltose is the smallest substrate capable of rapid hydrolysis.

Pullulan is a glucan consisting of alpha-maltotriose polymerized endwise through 1→6-bonds (2). Pullulanase is the name given to an extracellular enzyme of *Aerobacter aerogenes* that was found by Bender and Wallenfels (2) to cause an essentially quantitative hydrolysis of pullulan to maltotriose. Realizing that pullulan has a formal similarity to amylopectin and that its action was similar to that of the plant enzyme that splits the alpha-1→6-branch points of this polysaccharide (R-enzyme, 3), Bender and Wallenfels (2) tested pullulanase against

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amylopectin and observed the same increase in intensity of iodine stain and reducing power that characterizes R-enzyme action. Unlike R-enzyme (3), pullulanase is extremely easy to prepare in quantity and in a seemingly enzymatically pure form, simply by precipitation of the protein in an extracellular filtrate of *A. aerogenes* (2). As such it has great potential in the laboratory for the exploration of starch structure, and on the industrial scale for starch modification. This communication is an attempt to define the specificity of pullulanase and to make further comparison with R-enzyme.

Methods

Paper Chromatography. The solvents and spray reagents were as used by Peat *et al.* (4).

Polysaccharides and Oligosaccharides. The amylopectin and glycogen were prepared, respectively, from waxy maize and rabbit liver, as described by Schoch (5). Pullulan was prepared as by Bender *et al.* (6). Alpha-limit dextrins were purified from the mixture of dextrins formed when crystalline human salivary alpha-amylase acts on amylopectin (7,8) and were purified by chromatography on charcoal-Celite (8,9). Beta-limit dextrins of amylopectin and glycogen were prepared as by Whelan (10). Panose was prepared as by Smith and Whelan (11), isomaltose as by Taylor and Whelan (12), and 6-alpha-maltosylglucose and 6-alpha-maltotriosylglucose as by French *et al.* (1). The concentrations of all solutions of poly- and oligosaccharides were determined by acid hydrolysis to glucose (13), measured by its reducing power (14) or with glucose oxidase (15).

Enzymes. Pullulanase was prepared as by Bender and Wallenfels (2).[6] The cell-free filtrate of *A. aerogenes* was treated at 0°C. with acetone (−10°), stored for several hours at 0°, centrifuged, washed with cold acetone, and dried in a desiccator. Solutions of the enzyme were prepared by shaking 1 g. of enzyme powder in 25 ml. of 0.02*M* phosphate buffer, pH 6.8, for 18 hr. at 2°. The extract was centrifuged (10,000 rev./min. for 10 min.) and the solid re-extracted with 10 ml. of buffer for 2 to 3 hr. After the second extract was centrifuged, the supernatant solutions were combined. In some experiments the enzyme solution was dialyzed, before use, against 0.02*M* phosphate buffer, pH 6.8, for 18 hr. at 2°. This was to remove a trace of oligosaccharide-like material.

Crystalline beta-amylase was a twice-crystallized specimen from sweet potato, purchased from the Worthington Biochemical Corpora-

[6] An improved method of preparing pullulanase has been devised by K. Wallenfels and co-workers, yielding material of much higher specific activity. This will be published elsewhere.

tion, Freehold, N.J. Its activity was protected with human-serum albumin and reduced glutathione, as by Walker and Whelan (16), whose conditions for its use in hydrolyzing polysaccharides were observed. When the enzyme was used in high concentration to hydrolyze maltotriose, 2-amino-2-hydroxymethyl-1,3-propanediol (tris) was incorporated into the digests as an inhibitor (17) against a trace of alpha-glucosidase activity in the beta-amylase (18).

Measurement of Enzyme Activity. Pullulanase solution (0.1 ml.) was incubated with a mixture of 0.02*M* phosphate-citrate buffer (pH 5.0, 0.7 ml.) and 5% pullulan solution (0.2 ml.) at 30°. Samples of 0.1 ml. each were withdrawn after 10, 20, and 30 min., into a mixture of water (0.9 ml.) and Nelson reagent C (1 ml.) (19). Reducing power was then estimated as by Nelson (19), the reagent being calibrated against maltotriose. The reducing powers were corrected for "background" reduction, measured in a similar digest of pullulan and buffer, enzyme being omitted. There was a linear relation between amount of enzyme and weight of sugars liberated (calculated as maltotriose), provided that no more than 130 γ of maltotriose was present in the 0.1-ml. assay sample. The activity of the enzyme solution is expressed in units, one unit being the amount that liberates 1 μmole of maltotriose per min. in the prescribed 1 ml. digest (20). A typical activity level was 5.6 units of enzyme per ml. of pullulanase extract, made as described above.

Beta-amylase activity was measured as by Walker and Whelan (16), except that the temperature of incubation was 30°. The original units in which its activity was measured have been recalculated here in terms of the parameters recommended by the International Commission on Enzymes (20). One unit is now defined as the amount of enzyme catalyzing the release of 1 μmole of maltose per min. per ml. of digest at pH 4.8 and 30°C. This new unit is equal to 14 units on the old scale (21).

Enzyme-Substrate Digestions. All digestions were carried out at pH 5.0 and 35° and contained the following components (approx. final concentrations): 6 m*M* citrate buffer, pH 5.0, 0.5% poly- or oligosaccharide, pullulanase (0.4 units/ml.), and/or beta-amylase (73 units/ml.). Reducing power was measured with a copper reagent (14), or where oligosaccharides that would release glucose on splitting of a 1→6-bond were used, the glucose oxidase reagent (15) was employed.

Results and Discussion

Comparison of Pullulanase and R-Enzyme. In Table I are listed the degrees of beta-amylolysis of four polysaccharides, before and after pullulanase action. These results are compared with previously published

TABLE I

EXTENTS OF DEBRANCHING OF AMYLOPECTIN AND GLYCOGEN BY R-ENZYME AND PULLULANASE AS JUDGED BY THE INCREASE IN DEGREE OF BETA-AMYLOLYSIS

SUBSTRATE	CONVERSION INTO MALTOSE				
	Beta-Amylase Alone	Successive Actions of Debranching Enzyme and Beta-Amylase		Simultaneous Actions of Debranching Enzyme and Beta-Amylase	
		R-Enzyme	Pullulanase	R-Enzyme	Pullulanase
	%	%	%	%	%
Waxy-maize amylopectin	52	64	92	101	99
Waxy-maize amylopectin Beta-limit dextrin	0	73	99	...	103
Rabbit-liver glycogen	48	47	56	...	97
	40	..	..	40	...
Rabbit-liver glycogen Beta-limit dextrin	0	0	39	...	...

values obtained with R-enzyme as the agent for hydrolysis of branch linkages (3,22). There are similarities and differences between the enzymes. The similarities are with amylopectin. The polysaccharide is incompletely debranched by either enzyme acting alone, though pullulanase action is markedly more extensive than that of R-enzyme. Debranching of amylopectin beta-limit dextrin is much more complete than of amylopectin; by pullulanase it appears to be virtually complete. One or both of two effects could be operating here. The action of beta-amylase on amylopectin is to bring a "layer" of 1→6-linkages to the surface, and it is easy to imagine that they may now be more accessible to the debranching enzyme. Amylopectin has the dendritic structure originally proposed by Meyer and Bernfeld (23,24,25). The structure is most dense at the surface, decreasing in density as the center, the original nucleus, of the molecule is approached. One has to imagine, moreover, that a limit is imposed on the possible size of such a molecule, because the surface becomes too congested (26). It will be seen, therefore, that once the surface density of the amylopectin has been reduced, by beta-amylase, the task of pullulanase in penetrating the interior must become progressively easier, because the relative density of the branch points in space decreases. This factor, the spacing of the branch points, cannot by itself be the sole factor determining the ability of the enzyme to split the 1→6-links. It has already been shown for R-enzyme (8), and we have confirmed it for pullulanase, that doubly branched oligosaccharides in which no more than two glucose units separate the branch points are attacked. Pullulan itself, though not branched, also has only two 1→4-bonds separating each pair of

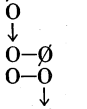
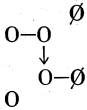
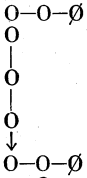
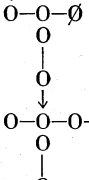
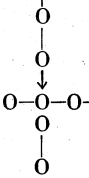
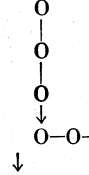
1→6-bonds, yet is split by pullulanase. This interlinkage distance is much lower than the relative average spacing between 1→6-links in amylopectin. One has to think of amylopectin on a larger scale and borrow the terminology of secondary and tertiary structures, as applied to protein macromolecules. This concept is seen to have validity when applied to glycogen and R-enzyme. The latter has no detectable action in increasing the degree of beta-amylolysis, either of glycogen itself or of the beta-dextrin formed in the presence of active beta-amylase (22, Table I). Yet once the secondary and tertiary structures of glycogen are disrupted by alpha-amylase, R-enzyme can exert its action (22). Pullulanase, by contrast, can effect a limited degree of hydrolysis of glycogen and has a markedly more extensive action on the beta-dextrin. This being so, it could be expected that if beta-amylase was allowed to act simultaneously with pullulanase, continually exposing new "beta-dextrins" in the inner regions of glycogen, the degradation should proceed to completion. This is what happened, as it did for amylopectin also (Table I). In the context of the catabolism of amylopectin *in vivo*, it will be seen that the debranching enzyme, to be fully effective, requires the assistance of an enzyme that will shorten the unit chains, presumably phosphorylase, just as the latter is required to assist the enzymatic complex of transglycosylase and amylo-1,6-glucosidase that splits the branch points of glycogen (16,27).

Substrate Specificity of Pullulanase. The specificity of R-enzyme was defined some years ago (28), before the range of oligosaccharides now available for such studies was prepared. It was concluded, sometimes on indirect evidence (29), that R-enzyme, to split a 1→6-bond, required that the substrate contain alpha-1→4-linkages in both portions of the molecule joined through the 1→6-bond; i.e., neither portion could be glucose. The smallest molecules found to be split by R-enzyme were the pentasaccharides G (Table II) (8,30) and 6^2-alpha-maltosyl-maltotriose (31). It must be noted that R-enzyme has been fractionated into two enzymes, one splitting branch points in amylopectin and not in oligosaccharides, the second having the opposite specificity (32). We do not yet know whether pullulanase can be so fractionated.

Pullulanase was tested on each of the oligosaccharides in Table II, and also on pullulan. The substrates were "equimolar" in concentration with respect to 1→6-bonds (6 m*M*). The initial rates of cleavage of the substrates were measured and are listed in Table II. The results were exactly those which would have been expected of R-enzyme. Oligosaccharides conforming to the structure alpha-maltodextrinyl-(1→6)-maltodextrin were split at rates of the same order as pullulan. Oligosaccharides containing glucose in one or both halves of the

TABLE II

RATES OF HYDROLYSIS OF OLIGOSACCHARIDES AND PULLULAN BY PULLULANASE

	OLIGOSACCHARIDE		RELATIVE RATE OF HYDROLYSIS
	Name	Structure	
			%
A	Isomaltose		0
B	6^2-alpha-Glucosylmaltose		0
C	6-alpha-Maltosylglucose		0
D	6^3-alpha-Glucosylmaltotriose		0
E	6-alpha-Maltotriosylglucose		0.06
F	6^2-alpha-Maltosylmaltose		23
G	6^3-alpha-Maltosylmaltotriose		55
H	6^3-alpha-Maltotriosylmaltotriose		91
I	6^3-alpha-Maltosylmaltotetraose		171
J	6^3-alpha-Maltotriosylmaltotetraose		112
K	6^3-alpha-Maltotetraosylmaltotriose		57
L	Pullulan		100

molecule were not split or, where splitting was noted (6-alpha-maltotriosylglucose, E, Table II), the rate relative to pullulan was so low that no significance could be attached to it. The enzyme preparation used was simply a cell-free filtrate and the hydrolysis of the 6-alpha-maltotriosylglucose could have been due to an enzymatic impurity.

It is clear that, like R-enzyme, pullulanase has as a minimum requirement, at least for rapid hydrolysis, that both of the components joined through the 1→6-bond shall be alpha-1→4-bonded glucose oligosaccharides. The smallest molecule conforming to this requirement is the tetrasaccharide F (Table II).[7] This was synthesized recently by Gwen J. Walker (33), who had already noted its hydrolysis by pullulanase, and to whom we are indebted for the sample used here. This tetrasaccharide, therefore, represents the smallest substrate conforming to the requirement for rapid hydrolysis by pullulanase. One might reasonably speculate that the same minimum requirement would also be found for R-enzyme.

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[7] An isomeric, so far unknown, tetrasaccharide, i.e. 6^1-alpha-maltosylmaltose, might also be a substrate.

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