INHIBITION OF PULLULANASE BY SCHARDINGER DEXTRINS

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1. Introduction

Earlier reports [1–3] have described the inhibition of sweet-potato β-amylase (1,4-α-glucan maltohydrolase, EC 3.2.1.2) and potato α-glucan phosphorylase (1,4-α-glucan:orthophosphate α-glucosyltransferase, EC 2.4.1.1) by the Schardinger dextrins, which are cyclic oligosaccharides consisting of 1,4-bonded α-glucose units without a chain end. The inhibition has been seen as logical in the context that the two enzymes act in exo fashion at the non-reducing chain ends of 1,4-bonded α-glucose polymers and has been explained on the basis of competition of the dextrins for the primer binding site of potato phosphorylase, and for the substrate binding site of β-amylase. It is now reported, in contrast, that the Schardinger dextrins are much more powerful inhibitors of an endo-acting amylopectin-hydrolyzing enzyme, pullulanase, whose hydrolytic role is the scission of 1,6-linkages.

2. Materials and methods

Crystalline pullulanase (pullulan 6-glucanohydrolase, EC 3.2.1.41) from *Aerobacter aerogenes* [4] was supplied by Dr. Christiane Mercier. Isoamylase (glycogen 6-glucanohydrolase, EC 3.2.1.68) from a species of *Cyrophaga* [5] was purified by ion-exchange chromatography on DEAE-cellulose, followed by molecular-sieve chromatography on Biogel P-60. *Aspergillus niger* glucoamylase (1,4-α-glucan glucohydrolase, EC 3.2.1.3) was prepared by Qureshi [6] using the method of Fleming and Stone [7]. A partly purified preparation of *Pseudomonas stutzeri* amylase (1,4-α-glucan maltotetraohydrolase, EC 3.2.1.60) was obtained by ammonium sulfate fractionation of a culture filtrate of the organism grown as described by Robyt and Ackerman [8]. Rabbit-muscle debranching enzyme (amylo-1,6-glucosidase, EC 3.2.1.33 –1,4-α-glucan: 1,4-α-glucan 4-α-glucosyltransferase, EC 2.4.1.25) was supplied to Professor W.J. Whelan by Dr. T.E. Nelson. Barley β-amylase and pig pancreatic α-amylase (1,4-α-glucan glucanohydrolase, EC 3.2.1.1.) were commercial samples obtained from Wallerstein Laboratories and the Sigma Chemical Company respectively. Sweetcorn pullulanase was prepared as discribed earlier [9].

Pullulan was produced by growth of *Pullularia pullulans* on sucrose [10], precipitated from the culture filtrate with ethanol, and purified before use by reprecipitation. Shellfish glycogen (Mann Research Laboratories) was 3-times reprecipitated with ethanol before use. Amylopectin (waxy maize starch) was prepared as described by Schoch [11] and its β-limit dextrin as by Whelan [12]. Potato amylose was purchased from Nutritional Biochemical Corporation. The Schardinger dextrins, cyclomaltohexaose and cyclomaltoheptaose, were prepared and purified as by French et al. [13].

Pullulanase activity was determined using enzyme digests (1.0 ml) containing pullulan (5.0 mg) and acetate buffer (pH 5.0, final concentration 100 mM) incubated at 37°C. Reducing sugars liberated were determined using the Nelson adaptation [14] of Somogyi's copper reduction method [15], calibrated against glucose. One unit of pullulanase is the amount which liberates 1 μmole of reducing sugar (measured as glucose) per minute under these conditions.

Descending paper chromatograms were developed

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at room temperature in the solvent system ethyl acetate:pyridine:water (10:4:3, by volume). Schardinger dextrins were detected in the developed chromatograms by exposure to iodine vapor. Reducing sugars were detected with the alkaline silver nitrate reagent [16].

Carbohydrate contents of substrate solutions and Schardinger dextrin solutions were determined using the phenol–sulfuric acid method [17]. Glucose was determined specifically by using glucose oxidase reagent [18].

3. Experimental procedures

3.1. *Determination of pullulanase inhibition by various concentrations of cyclomaltohexaose and cyclomaltoheptaose*

Enzyme digests were prepared containing pullulan (5 mg), acetate buffer (pH 5.0, final concentration 100 mM), pullulanase (0.012 unit) and various concentrations of cyclomaltohexaose and cyclomaltoheptaose [in the ranges 5.9–35.4 μg/ml (cyclomaltohexaose) and 0.94–4.7 μg/ml (cyclomaltoheptaose)] and the amounts of reducing sugars liberated determined after incubation at 37°C for suitable lengths of time. The inhibition of pullulanase action on amylopectin β-limit dextrin by cyclomaltohexaose and cyclomaltoheptaose (final concentrations 23.6 and 2.82 μg/ml respectively) was determined in a similar manner.

3.2. *Determination of pullulanase inhibition by other α-D-glucans and oligosaccarides*

The effect of amylose, glycogen and linear maltodextrins on pullulanase activity was tested using enzyme digests as above, but with the Schardinger dextrins replaced by these polysaccharides or oligosaccharides at concentrations up to 2.5 mg/ml.

3.3. *Determination of the effect of cyclomaltohexaose and cyclomaltoheptaose on other starch-degrading enzymes*

The effect of cyclomaltohexaose and cyclomaltoheptaose at concentrations as high as 9.4 mg/ml was tested on the degradation of soluble starch by pig pancreatic α-amylase, barley β-amylase, *Pseudomonas stutzeri* amylase and *Aspergillus niger* glucoamylase. The effects of the cyclic dextrins on the activity of isoamylase on glycogen, and rabbit-muscle debranching enzyme on glycogen phosphorylase limit dextrin, and on the activity of sweet-corn pullulanase were also examined. All enzyme digests contained the appropriate buffer and samples were removed for measurement of reducing sugars in all cases except rabbit-muscle debranching enzyme where the release of glucose was measured. Control experiments showed that the cyclodextrins had no effect on the glucose oxidase procedure used.

3.4. *Measurement of the inhibitor constants for Shardinger dextrin inhibition of pullulanase*

Initial rates of enzyme action were determined in digests (1.0 ml) containing pullulan (1.0–8.0 mg), acetate buffer (pH 5.0, final concentration 50 mM), pullulanase (9×10^{-3}IU) with and without cyclomaltohexaose (23.5 μg/ml) or cyclomaltoheptaose (4.7 μg/ml). The inhibitor constants were determined from Lineweaver – Burk double reciprocal plots (fig. 1.).

4. Results and discussion

Table 1 shows the inhibition of *Aerobacter aerogenes* pullulanase by various concentrations of cyclomaltohexaose and cyclomaltoheptaose. These dextrins are seen to be extremely powerful inhibitors of the enzyme and the amount required to give a certain extent of inhibition is several orders of magnitude less than that required to give the same inhibition of sweet-potato β-amylase [1,2] or potato phosphorylase [3]. Inhibition of pullulanase action on amylopectin β-limit dextrin is of similar magnitude to that on pullulan, showing that the Schardinger dextrins have a true effect on the enzyme, rather than on the substrate. In contrast to the situation with the inhibition of sweet-potato β-amylase [2], in this case the larger ring dextrin is the more powerful inhibitor. Lineweaver – Burk double reciprocal plots of the reaction kinetics in the presence of the inhibitors showed that the inhibition by both cyclodextrins is of a competitive type, as for the inhibition of other enzymes by these compounds [1,3]. The inhibitor constants for cyclomaltohexaose and cyclomaltoheptaose are 2.91×10^{-6} M and 2.15×10^{-7} M. These values

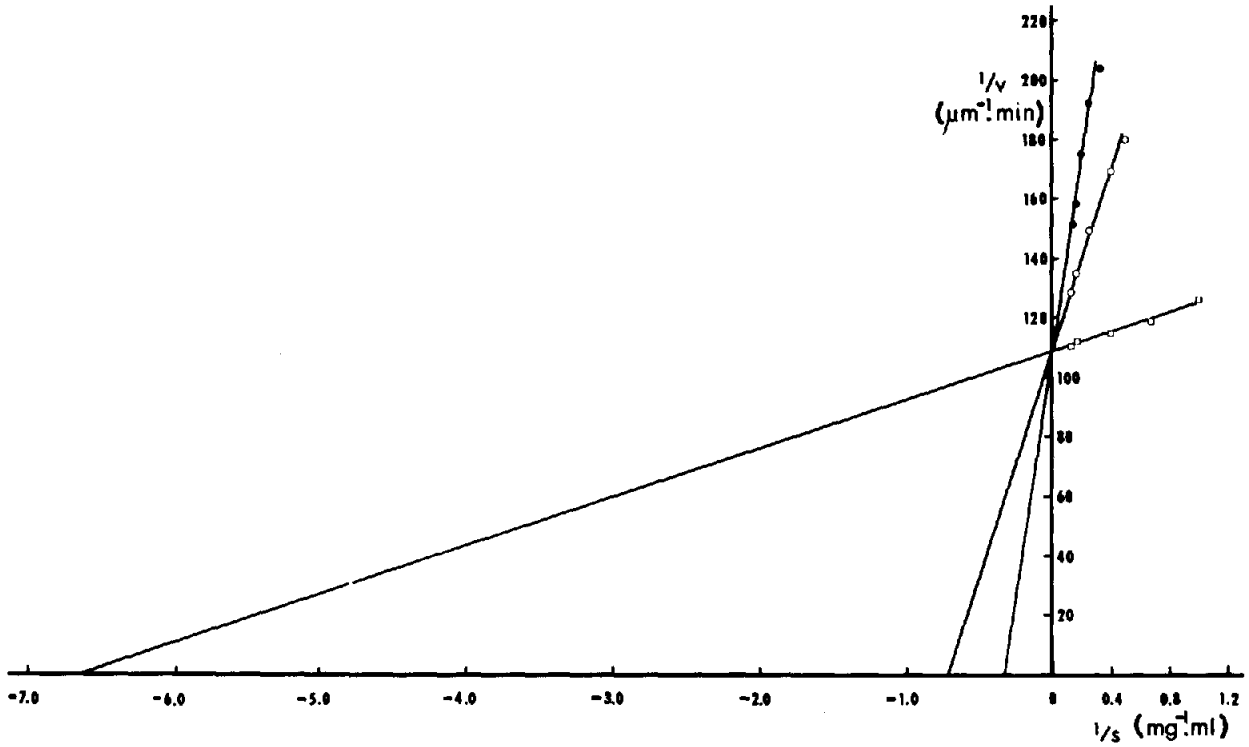

Fig. 1. Lineweaver – Burk plots for action of *Aerobacter aerogenes* pullulanase on pullulan in the absence of Schardinger dextrins (□-□), and in the presence of cyclomaltohexaose (○-○) and cyclomaltoheptaose (●-●). The concentrations of cyclomaltohexaose and cyclomaltoheptaose were 23.5 μg/ml (2.41×10^{-5} M) and 4.7 μg/ml (4.14×10^{-6} M) respectively. Experimental details are given under Materials and methods.

Table 1
Inhibition of *Aerobacter aerogenes* pullulanase by cyclomaltohexaose and cyclomaltoheptaose.

Cyclomaltohexaose		Cyclomaltoheptaose	
Concentration (μg/ml)	Inhibition* (%)	Concentration (μg/ml)	Inhibition* (%)
0	0	0	0
5.9	11	0.94	9
11.8	21	1.88	22
17.7	24	2.82	33(39)
23.6	28(50)	3.76	42
29.5	33	4.70	44
35.4	39		

* Determined with pullulan as substrate, except for the values used in parentheses which were determined towards amylopectin β-limit dextrin.

may be compared with 3.1×10^{-4} M and 2.0×10^{-3} M for cyclomaltohexaose and cyclomaltoheptaose as inhibitors of sweet-potato β-amylase [2].

The corresponding linear oligosaccharides (maltodextrins), used at concentrations of 0.25 mg/ml do not inhibit pullulanase. Neither do glycogen nor amylopectin cause significant inhibition of the enzyme, even at concentrations as high as 2.5 mg/ml. At that concentration, however, amylose causes about 25% inhibition. It is not known whether the mechanism of inhibition by amylose is related to that caused by the Schardinger dextrins. The inhibition of pullulanase by amylose is, however, of significance in that it will mitigate against the use of pullulanase to debranch the amylopectin component in the conversion of normal starch into an artificial high amylose starch [19].

Table 2 shows the effect of Schardinger dextrins on

Table 2
Inhibition of starch-degrading enzymes other than pullulanase by cyclodextrins.

Enzyme	Cyclo-dextrin*	Concen-tration (mg/ml)	Inhibi-tion (%)
Hog pancreatic α-amylase	α	9.4	14
Barley β-amylase	α	9.4	66
Pseudomonas stutzeri amylase	α	9.4	0
Aspergillus niger glucoamylase	α	9.4	15
Cytophaga isoamylase	α	6.25	2
	β	6.25	0
Rabbit-muscle debranch-ing enzyme	α	6.25	59
	β	6.25	48
Sweet-corn pullulanase	α	58.8×10^{-3}	54
	β	11.75×10^{-3}	76

* α Denotes cyclomaltohexaose.
β Denotes cyclomaltoheptaose.

other starch-degrading enzymes. The only noteworthy inhibitions, other than the expected inhibition of barley β-amylase, are of rabbit-muscle debranching enzyme and sweet-corn pullulanase. Inhibition of the latter enzyme by the Schardinger dextrins is further support for the similarity we have proposed [9] between the plant and bacterial pullulanases. By contrast, another enzyme that directly debranches amylopectin, *Cytophaga* isoamylase [5], was not significantly inhibited. Thus there is no relation between the action pattern of a starch-degrading enzyme (i.e. endo or exo mode of attack) and its susceptibility to inhibition by the Schardinger dextrins, since enzymes of both types may be inhibited or uninhibited. Since pullulanase is known to have some unusual transferase properties [22] it was considered important to confirm that enzyme action was truly inhibited, rather than being changed from hydrolysis to transfer, with resultant formation of substituted cyclomaltodextrins, a process that would not be detected by reducing power measurements. However, paper chromatographic examination of an enzyme digest containing pullulanase, cyclodextrin and pullulan did not reveal the presence of any substituted cyclodextrins.

The above findings have some important practical implications. It will be necessary to take into consideration the inhibitory effect of the cyclic dextrins in experiments where 6-substituted Schardinger dextrins are used as test substrates for plant and microbial pullulanases, since any enzyme action will result in production of a most powerful enzyme inhibitor. This may be one reason why only very low action of a cereal debranching enzyme (now considered to be a typical pullulanase) was observed using α-maltosyl cyclomaltohexaose as substrate [23]. The same holds true when glucosyl substituted Schardinger dextrins are used as substrates for mammalian glycogen debranching enzymes [24]. Indeed, the substituted Schardinger dextrins themselves may be inhibitory to the enzymes and use of such substrates may be undesireable unless the inhibition is outweighed by considerations of substrate specificity. Examination of the effect of the Schardinger dextrins on a debranching enzyme may be a facile method for classifying it as being of the isoamylase or pullulanase type, in view of the differences in the inhibition of these two types of debranching enzymes. Similarly the selective cyclodextrin inhibition of pullulanase may be of assistance in detecting this enzyme in mixtures of starch-splitting enzymes.

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