Vol. 166, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1990 Pages 126-132

SUBSTRATE COMPETITION AND SPECIFICITY AT THE ACTIVE SITE OF AMYLOPULLULANASE FROM *CLOSTRIDIUM THERMOHYDROSULFURICUM*

Saroj Mathupala[1], Badal C. Saha[3], and J. Gregory Zeikus[1,2,3]

Department of Biochemistry[1] and Department of Microbiology and Public Health[2], Michigan State University, East Lansing, MI 48824

Michigan Biotechnology Institute[3], Lansing, MI 48909

Received October 19, 1989

A highly thermostable pullulanase purified from *Clostridium thermohydrosulfuricum* strain 39E displayed dual activity with respect to glycosidic bond cleavage. The enzyme cleaved α-1,6 bonds in pullulan, while it showed α-1,4 activity against malto-oligosaccharides. Kinetic analysis of the purified enzyme in a system which contained both pullulan and amylose as the two competing substrates were used to distinguish the dual specificity of the enzyme from the single substrate specificity known for pullulanases and α-amylases.

Clostridium thermohydrosulfuricum strain 39E produces a highly thermostable pullulanase activity when grown on starch (1). This enzyme was homogeneously purified and partially characterized in terms of MW, pI, pH stability, and thermal stability (2).

Pullulanase (pullulan 6-glucanohydrolase, EC 3.2.1.41) is a debranching enzyme that specifically cleaves α-1,6 links in starch, amylopectin, pullulan and related oligosaccharides (3), while α-amylases (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) hydrolyse the α-1,4 linkages (4). Pullulanases do not show activity against linear (α-1,4-linked) oligosaccharides, and α-amylases show no activity against pullulan.

The novel pullulanase of *C. thermohydrosulfuricum* strain 39E described in this communication showed α-1,4 as well as α-1,6 cleavage activity against amylose and pullulan, respectively. Detailed kinetic studies with the homogeneously purified enzyme were performed with pullulan, amylose, and linear low MW oligosaccharides in order to biochemically evaluate the active site and to propose a name for this newly recognized enzyme activity.

METHODS

Materials. All chemicals used were obtained from either Sigma Chemical Company (St. Louis, MO) or Aldrich Chemical Company (Milwaukee, WI).

Bacterial strains and growth conditions. *Clostridium thermohydrosulfuricum* strain 39E (ATCC 33223) was used as the source of pullulanase. The organism was grown at 60°C under

0006-291X/90 $1.50

anaerobic conditions in TYE medium (5) with 1% (w/v) soluble starch as the substrate. Isolation and purification of the enzyme was performed as described previously (2).

Enzyme assay. 160 microliters of pullulan solution (1.25% w/v in 50 mM NaOAc buffer, pH 6.0, containing 5 mM $CaCl_2$) and 40 microliters of enzyme solution were mixed and incubated at 60°C for 30 min. The reaction was stopped by adding 0.8 ml of DNS solution (6) and heated in a boiling water bath for 15 min. The samples were cooled in ice and the absorbance of the reaction solution was measured at 640 nm. One unit of pullulanase activity is defined as the amount of enzyme which produced 1 micromole of reducing sugar (with glucose as standard) per minute under the above assay conditions.

Competitive inhibition kinetics with mixed alternative substrates. The enzyme recognizes both pullulan and amylose as substrates. It cleaves essentially the α-1,6 bonds in pullulan, while in amylose, the α-1,4 bonds are cleaved. In this experiment both substrates were present as mixed alternative substrates (as outlined by Segel [7]), and was used to investigate the following possibilities:

1. If both activities are due to an enzyme complex of a pullulanase and an α-amylase, or if both activities occurred within the same enzyme, but at two individual active centers non-interacting with each other, then the initial velocity obtained for product formation with both substrates present should be approximately the sum of the individual initial velocities when either of the substrates is present.

$$v = v_P + v_A$$

$$v = \frac{V_A \cdot [A]}{Km_A + [A]} + \frac{V_P \cdot [P]}{Km_P + [P]}$$

where [A] = amylose concentration
[P] = pullulan concentration
V, maximum velocity for each substrate
v, initial velocity
Km, apparent Michaelis constant for each substrate

2. If both activities are due to a single enzyme having a single active center, or two active sites negatively interacting with each other, then the initial velocity obtained with both substrates present will be less then the sum of the individual velocities.

$$v < v_P + v_A$$

$$v = \frac{V_A \cdot [A]}{Km_A\,(1+[P]/Km_P)+[A]} + \frac{V_P \cdot [P]}{Km_P(1+[A]/Km_A)+[P]}$$

RESULTS AND DISCUSSION

Incubation of the enzyme with low MW oligosaccharides, (maltotetraose to maltoheptaose) resulted in the substrates being degraded into units of maltotriose and residual sugars (i.e., glucose or maltose), depending on the parent oligosaccharide subjected to enzymatic action (Table 1), demonstrating that the enzyme had no activity against maltotriose. Maltotetraose was a poor substrate for the enzyme and maltose was not detected as its hydrolysis product. These results reflect an important deviation from known α-amylase activity and pullulanase activity, as maltotriose, as well as other oligosaccharides, would have been

TABLE 1. Reaction products of amylopullulanase from *C. thermohydrosulfuricum* strain 39E on low MW oligosaccharides[a]

Substrate	End Products					
	G_1	G_2	G_3	G_4	G_5	G_6
Maltotriose	--	--	++	--	--	--
Maltotetraose[b]	++	--	++	++	--	--
Maltopentaose	--	++	++	--	--	--
Maltohexaose	--	--	++	--	--	--
Maltoheptaose	++	--	++	--	--	--

Major products observed are shown by the positive sign.

[a]Solutions of 1% (w/v) maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose were incubated at 60°C with purified enzyme (0.05 U/ml). Products were analyzed after 72 hours by HPTLC (Whatman HP-K). Plates were developed with n-BuOH:EtOH:H_2O (3:2:2, v/v) at 25°C and the products detected with a mixture of 0.2% (w/v) orcinol in MeOH and 20% (v/v) H_2SO_4 in MeOH (1:1, v/v).

[b]Maltotetraose is a very poor substrate for the enzyme and the products were observed only after long-term reaction.

finally degraded into glucose and maltose by saccharifying α-amylase, and no activity would have been shown towards any of these α-1,4-linked oligosaccharides by pullulanase.

The enzyme from *C. thermohydrosulfuricum* strain 39E showed broad range substrate specificity with regard to high MW polysaccharides. The final reaction products obtained were maltose, maltotriose, and maltotetraose (Table 2). The only product obtained upon incubation of the enzyme with pullulan was maltotriose, demonstrating that the enzyme shows typical pullulanase activity.

The present data indicate that the pure enzyme has both "α-amylase" and "pullulanase-like" activity, and its own unique mode of action. In general, these data show that the mode of action of the enzyme from *C. thermohydrosulfuricum* strain 39E is different from that

TABLE 2. Reaction products of amylopullulanase from *C. thermohydrosulfuricum* strain 39E on high MW polysaccharides[a]

Substrate	End Products					
	G_1	G_2	G_3	G_4	G_6	G_9
Pullulan	--	--	100	--	--	--
Amylose	--	37	47	16	--	--
Amylopectin	--	36	36	28	--	--
Soluble starch	--	39	39	22	--	--
Mammalian glycogen	--	22	47	31	--	--
Oyster glycogen	--	17	50	33	--	--

[a]Solutions of 1% (w/v) pullulan, amylose, amylopectin, mammalian glycogen, oyster glycogen and soluble starch (pH 6.0) were incubated at 60°C with purified enzyme (0.05 U/ml). Samples were withdrawn after 216 hrs and heated at 100°C for 15 min for enzyme inactivation. The reaction products were analyzed by HPLC for sugars (2).

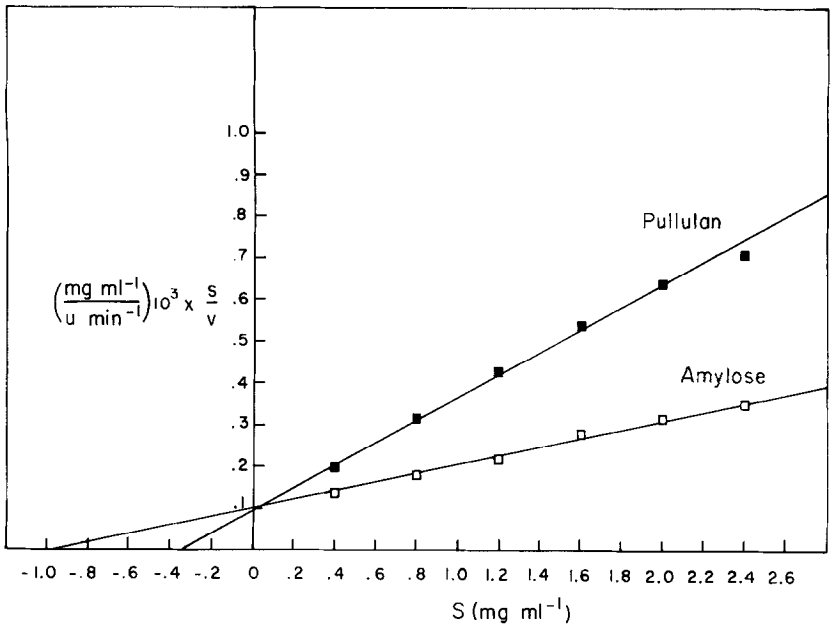

Fig. 1. Km^{app} determination. Pullulan (■) and low MW amylose (□) at the concentrations indicated were incubated with purified enzyme at 60°C.

previously reported for pullulanase and α-amylase, in terms of bond cleavage specificity as well as product formation.

The Km^{app} for pullulan (average MW 50,000) and low MW amylose (MW 4,100) were obtained at substrate concentrations between 0.4 mg/ml to 2.4 mg/ml at 0.4 mg/ml increments (Fig. 1). The dependence of the rate of pullulan and amylose hydrolysis on the substrate concentration followed Michaelis-Menten kinetics. The apparent Km for pullulan (average MW 50,000) and amylose (MW 4,100) as determined from the Hanes-Woolf plot were 0.35 mg/ml and 1.00 mg/ml, respectively (Fig. 1). The apparent k_{cat} for pullulan was 16,000 min^{-1}.

Kinetic experiments on competitive inhibition with mixed alternative substrates were performed at pullulan concentrations of 0.4 to 2.4 mg/ml at 0.8 mg/ml increments, while amylose concentrations were varied from 0.6 to 3.0 mg/ml at 0.6 mg/ml increments. Initial velocities were determined for each combination of pullulan and amylose at the different concentrations. The initial velocity was plotted against the total substrate concentration in a S/V versus S plot (where S is the total substrate concentration), and is shown in Fig. 2. For clarity, only two sets of data with amylose at 0.4 mg/ml and 2.4 mg/ml with varying pullulan concentrations are shown. The initial velocities obtained closely followed the theoretical plot for case 2 (as described in Methods), where the observed velocity was less than the cumulative value of the individual initial velocities obtained for the two substrates. This demonstrates competition between the two substrates used, indicating that the enzyme possesses an active

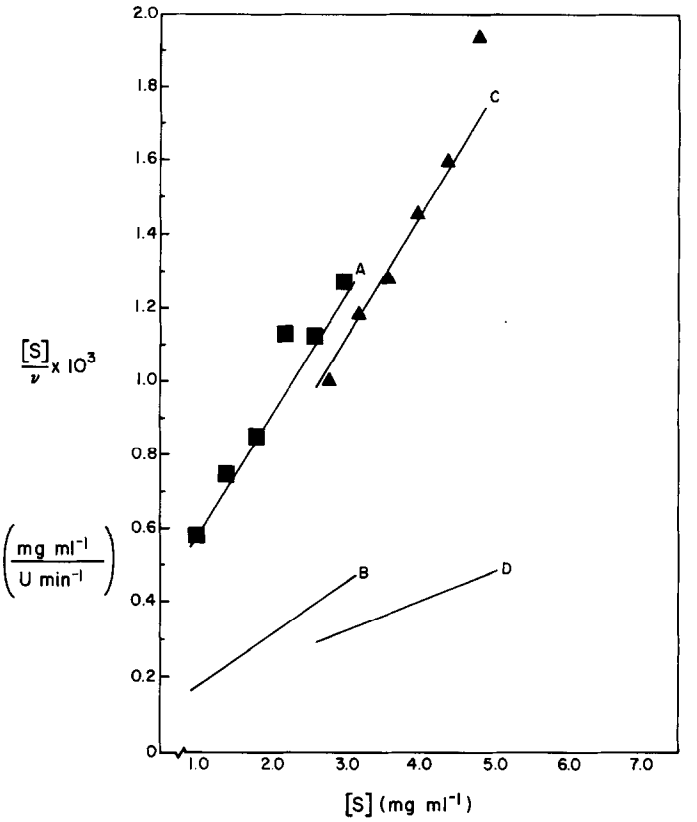

Fig. 2. Kinetics of competitive inhibition with mixed substrates. The solid lines A and C indicate the theoretical plots for competitive inhibition at amylose concentrations of 0.6 and 2.4 mg/ml, respectively. Lines B and D are the theoretical plots for absence of inhibition at the same respective amylose concentrations. Pullulan was used at concentrations of 0.4, 0.8, 1.2, 1.6, 2.0, 2.4 mg/ml. For clarity, only two sets of data points were used in the above plot. (■) and (▲) are the practical data points obtained at 0.6 and 2.4 mg/ml amylose concentrations. All reaction mixtures contained 5% (v/v) dimethyl sulfoxide for solubility of amylose.
[S] = [A] + [P], where S is the total substrate concentration. A and P are the concentrations of amylose and pullulan, respectively.

site for cleavage of both α-1,6 and α-1,4-linked substrates. This apparent competition between two substrates has been used by several authors as proof of a given enzyme having a single active center with two different modes of activity (8-12). However, as the same result can occur under certain conditions for an enzyme with two active centers (8), the results obtained for the enzyme from *C. thermohydrosulfuricum* strain 39E can only be used as positive proof for both activities belonging to the same enzyme.

The first description of pullulanase activity was an enzyme from *Klebsiella pneumoniae* (13), which specifically hydrolyses α-1,6 glycosidic linkages of pullulan to yield maltotriose. Other pullulan degrading enzymes (e.g., neopullulanase) which specifically cleave α-1,4 glycosidic bonds in pullulan to yield panose (14) and isopanose (15) have been reported.

This is the first detailed kinetic and substrate hydrolysis studies reported on novel pullulanases that cleave α-1,6 and α-1,4 bonds. Pullulanases possessing both α-1,6 and α-1,4 activity have been isolated from *Thermoanaerobium* Tok 6-B1 (16, 17), *T. brockii* (18), *Clostridium thermosulfurogenes* (19), and *C. thermohydrosulfuricum* strain E101-69 (20, 21). The enzyme from *T. brockii* was reported to hydrolyze starch into various sized oligomers, while the *Thermoanaerobium* strain Tok 6-B1 enzyme acted upon starch, amylopectin and amylose to yield predominantly maltose and maltotriose. Maltotetraose was completely hydrolysed to maltose by *Thermoanaerobium* Tok 6-B1 enzyme (16), and the enzyme was found to hydrolyse low MW oligosaccharides at two glucose residues away from a terminal, and release maltose as the product (17). The enzyme isolated from *C. thermohydrosulfuricum* strain E101-69 was in two forms of MW over 300,000, and the possibility of the presence of a third form was suggested (21). Each form was capable of hydrolysing both pullulan and amylose but oligosaccharide hydrolysis studies were not reported. In contrast, the pullulanase described in this paper was of monomeric form with a MW of 136,500.

In comparison to the enzyme from *Thermoanaerobium* Tok 6-B1, *C. thermohydrosulfuricum* strain 39E pullulanase produced maltose, maltotriose, and maltotetraose upon reaction with high MW polysaccharides. Also, it hydrolysed low MW oligosaccharides three glucose units away from a terminal, released maltotriose as the product, and did not form maltose from maltotetraose. These results represent significant differences between the pullulanases from *Thermoanaerobium* Tok 6-B1 and *C. thermohydrosulfuricum* strain 39E.

Kinetic studies of the homogeneous enzyme from *C. thermohydrosulfuricum* strain 39E on low MW amylose and pullulan as competing substrates, gave further proof that both activities resided on the same enzyme and putatively within the same active site. Therefore, the enzyme described here as amylopullulanase higher affinity and activity toward pullulan than amylose; and, it shows unique characteristics with regard to substrate utilization as well as product formation. In light of these results, it is now necessary to distinguish pullulanases which possess only α-1,6 cleavage activity from amylopullulanases that contain both α-1,6 and α-1,4 cleavage activity. Thus, we propose the enzyme commission use the term amylopullulanase to describe those enzymes which act on starch and cleave both α-1,6 bonds in pullulan and α-1,4 bonds in amylose.

Acknowledgment: We thank Dr. Susan Lowe for her help in preparing the manuscript, useful discussion, advice and encouragement.

REFERENCES

1. Hyun, H. H., and Zeikus, J. G. (1985) Appl. Environ. Microbiol. 49, 1168-1173.
2. Saha, B. C., Mathupala, S. P., and Zeikus, J. G. (1988) Biochem. J. 242, 343-348.
3. Abdullah, M., Catley, B. J., Lee, E. Y. C., Robyt, J., Wallenfels, K., and Whelan, W. J. (1966) Cereal Chem. 43, 111-118.

4. Thoma, J. A., Spradlin, J. E., and Dygert, S. (1971) In The Enzymes (P. D. Boyer, Ed.), Vol. V, pp. 115-189. Academic Press, New York.
5. Ng, T. K., Ben-Bassat, A., and Zeikus, J. G. (1981) Appl. Environ. Microbiol. 41, 1337-1343.
6. Miller, G. L. (1959) Anal. Biochem. 31, 426-428.
7. Segel, I. H. (1975) In Enzyme Kinetics, pp. 113-118. John Wiley and Sons, New York.
8. Hiromi, K., Hamazu, Z., Takahashi, K., and Ono, S. (1966) J. Biochem. 59, 411-418.
9. Sakano, Y., Hiraiwa, S., Fukushima, J., and Kobayashi, T. (1982) Agric. Biol. Chem. 46, 1121-1129.
10. Guranowski, A., and Schneider, Z. (1977) Biochim. Biophys. Acta 482, 145-158.
11. Walker, D. E., and Axelrod, B. (1978) Arch. Biochem. Biophys. 187, 102-107.
12. Plant, A. R., Clemens, R. M., Morgan, H. W., and Daniel, R. M. (1987) Biochem. J. 246, 537-541.
13. Bender, H., and Wallenfels, K. (1961) Biochem. Z. 334, 79-97.
14. Kuriki, T., Okada, S., and Imanaka, T. (1988) J. Bacteriol. 170, 1554-1559.
15. Sakano, Y., Matsuda, N., and Kobayashi, T. (1971) Agric. Biol. Chem. 35, 971-973.
16. Plant, A. R., Clemens, R. M., Daniel, R. M., and Morgan, H. W. (1987) Appl. Microbiol. Biotechnol. 26, 427-433.
17. Plant, A. R., Clemens, R. M., Morgan, H. W., and Daniel, R. M. (1987) Biochem. J. 246, 537-541.
18. Coleman, R. D., Yang, S. S., and McAllister, M. P. (1987) J. Bacteriol. 169, 4302-4307.
19. Madi, E., Antranikian, G., Ohmiya, K., and Gottschalk, G. (1987) Appl. Environ. Microbiol. 53, 1661-1667.
20. Melasniemi, H. (1987) Biochem. J. 246, 193-197.
21. Melasniemi, H. (1988) Biochem. J. 250, 813-818.