Phytochemistry, 1979, Vol. 18, pp. 765–769. © Pergamon Press Ltd. Printed in England. 0031-9422/79/0501-0765 $02.00/0

THE ACTIVE SITE OF YEAST ENDO-POLYGALACTURONASE CONTAINS SEVEN SUBSITES

JOHN H. MCCLENDON
School of Life Sciences, University of Nebraska, Lincoln, NB 68588, U.S.A.

(*Revised received* 17 *August* 1978)

Key Word Index—*Saccharomyces fragilis*; fungi; galacturonan; polygalacturonase; size of active site.

Abstract—The rate of hydrolysis of oligomers by the endopolygalacturonase of yeast is in the order: heptamer > hexamer > pentamer > tetramer. This suggests that the active site accommodates at least 7 units. Since the heptamer disappears concurrently with the bulk of larger oligomers, the maximum number of units appears to be 7. The release of labelled (unsaturated, or ^3H labelled and reduced) end units from larger substrate is interpreted to indicate that the enzyme interacts with 3 saccharide units toward the reducing end from the bond to be broken, and with 4 units toward the non-reducing end. The relative affinities for the enzyme of saccharide units in various positions are unequal, as indicated by the very low relative rate of monomer production from the hydrolysis of hexamer and pentamer, and the apparently unequal probability of two other modes of hexamer hydrolysis [(tetramer + dimer) = 2.5 (trimer + trimer)].

INTRODUCTION

Rexová-Benková [1] has proposed a model for the active site of an endopolygalacturonase (endo-D-galacturonanase, EC 3.2.1.15; endo-PG) from *Aspergillus niger*, and Rexová-Benková and Markovic have extended this analysis to a variety of other endo-PG's in a comprehensive review [2]. The endo-PG from *Saccharomyces fragilis* was grouped with that from *A. niger* in having an active site which interacted with 4 galacturonic acid subunits of the galacturonan chain. This model is thus similar in principle to the structure known to exist in lysozyme [3]. The term *subunit* is applied to the enzyme without implying that there is any structural subunit in the protein comparable to the glycoside units in the substrate. The evidence was further interpreted to suggest that the enzyme breaks the polysaccharide chain between the first and second subunits, counting from the reducing end [1, 2].

I report here data on the endo-PG from *S. fragilis* (yeast endo-PG) which indicate that the active site must be larger than this, probably of 7 subunits. In contrast with most previous endo-PG studies, the amount of the oligomers produced in hydrolysis of galacuronan (pectate) has been determined quantitatively following gel-filtration chromatography, instead of PC, which permitted the assay for the disappearance of the large oligomers produced during the first, rapid phase of reaction.

The main assumption is that the rate of hydrolysis may be determined by two semi-independent features of the enzyme, the attachment of the substrate to the catalytic site and the subsequent hydrolysis. With a long, flexible polysaccharide, the act of catalysis may depend on the interaction of as few as two saccharide units with the enzyme, but the attachment of the substrate to the enzyme may be greatly enhanced by saccharide subunits distant from the catalytic site, thereby permitting a faster net rate of hydrolysis. (With yeast endo-PG, dimer is not attacked, nor is there any evidence for transferase activity.) During the course of reaction of endo-PG on pectate, large amounts of high MW oligomers accumulate; the order of their subsequent disappearance is taken to be a measure of the relative affinities of the oligomers for the enzyme. In previous work [2], non-productive complexes of the trimer with enzyme have been shown to inhibit the hydrolysis of tetramer; dimer did not inhibit. In this study, the slow hydrolysis of tetramer was not observed, and inhibition by non-productive complexes should not change the relative order of hydrolysis.

A model is proposed on the assumption that any oligomer, which is attacked at a slower rate than polymer, is smaller than the overall active site of the enzyme. This is not necessarily true, but gives the maximum number of subunits. The model is tested, however, by the examination of the rates of production of labelled oligomers from substrate that had been labelled on either end, during the early, rapid, linear phase of reaction (in contrast to the hydrolysis of smaller oligomers). Within the limits of uncertainty of the effects of the labelling alterations, these results confirm the model proposed.

RESULTS AND DISCUSSION

Table 1 summarizes the general course of reaction by yeast endo-PG, as shown by several customary measurements plus the gel-filtration chromatography. The oligomers up to the heptamer were resolved in the eluates, while the oligomers from octamer to a DP of *ca* 30 were resolved from the polymer but not from each other. The rapid decrease in viscosity and amount of acid precipitate contrast with the more modest increase in reducing groups, as is characteristic of endo-PG. Reducing group production was almost linear until 9 hr, after which the rate rapidly declined. The decrease in polymer is not exponential like the fall in viscosity, but is linear until about 3 hr. The amount of large oligomer

Table 1. Progress of hydrolysis by yeast endo-PG at pH 5.5

Time (hr)	Acid ppt.	Viscosity* (%)	Reducing groups (mmol/l.)	(mmol/l./hr)	Distribution of substrate and product (%)† DP > 30	DP 7-30	DP 1-6
0	white gel	100	0	1.36	92	6	2
0.5	+ + +	50	0.68	1.39	79	16	5
1.25	+	17	1.72	1.38	63	28	8
2	(+)	6	2.76	1.34	36	44	20
4	—	0	5.45	1.07	7	38	55
9			10.8	0.16	2	11	87
30			14.2	0.03	2	12	85
72			15.6‡		2	5	93

* (Flow time) − (flow time at 72 hr), as determined with a Cannon–Fenske viscometer, expressed as % of the initial viscosity.
† Wt % of uronic acid units.
‡ i.e. Less than 30% of the value calculated for complete hydrolysis of 1% pectate.

fraction is seen to increase until it is as abundant as the remaining polymer, and then it is hydrolysed. Evidently, large oligomers have an affinity for the enzyme equal to that of polymer. Monomer and oligomers up to hexamer, treated as a group, are released rapidly only after a lag of *ca* 1 hr, but eventually account for nearly all of the original substrate. A small part of the polymer is resistant to endo-PG; this contains most or all of the nongalacturonide in the preparation [4].

Table 2 shows data from these same experiments, with the individual small fragments detailed, after most of the polymer had disappeared. At 4 hr, tetramer, pentamer and hexamer are present in *ca* equimolar amounts, with lesser amounts of heptamer, trimer and especially dimer and monomer. At this time the heptamer has already begun to decrease, along with the decrease of the whole large-oligomer fraction. Between 4 and 9 hr, change is essentially accounted for by the fragmentation of heptamer and larger substrate, with the production of pentamer, tetramer and trimer. After 30 hr, hydrolysis is only *ca* 2% of the initial rate, and change is chiefly marked by the hydrolysis of pentamer, slight hydrolysis of tetramer, and increase in trimer, dimer and monomer. Between 9 and 30 hr, the changes of resolved oligomers are the greatest, with decreases in hexamer and pentamer and increases in smaller fragments.

It should be noted that hydrolysis of heptamer, even in the presence of an excess of hexamer, is much faster than that of hexamer (between 4 and 9 hr, −70% of heptamer, −15% of hexamer). Likewise, the hydrolysis of hexamer is more rapid than that of pentamer (between 9 and 30 hr, −88% of hexamer, −52% of pentamer), and the tetramer is hardly attacked during the 72 hr of observation. It is unlikely that these results are due to differences in rates of production rather than hydrolysis, since this would require that the smaller oligomers be produced at higher rates than the large ones, which is not the case (Table 1).

Rexová-Benková [1] reported that the reaction velocities of the *Aspergillus* enzyme on defined substrates were: dimer (0), trimer (0.007), tetramer (0.132), pentamer (0.448), and hexamer (1.54 μ equivalent/min/mg enzyme). My results confirm this order. Using yeast endo-PG, Demain and Phaff [5] reported that the tetramer is hydrolysed *ca* 50 times faster than trimer and their data indicate that the pectate polymer is hydrolysed *ca* 80 times faster than the tetramer at pH 5, but only *ca* 10 times faster at pH 3.3, the optimum for hydrolysis of tetramer and trimer. In my studies, the hydrolysis of trimer was not observed, because of the low intrinsic rate as well as the unfavorable pH.

Rexová-Benková [1] concluded that there was an active site of 4 subunits, based upon the equality of the *maximum* velocities of reaction on tetramer and pentamer, ignoring the differences in rates (above) and the smaller K_m for pentamer than tetramer. If one accepts the argument that a substrate which is hydrolysed more slowly than one of infinite dimensions therefore fails to fully occupy the active site of the enzyme [1, 2], it is evident from the prior work cited that the active site of *Aspergillus*

Table 2. Oligosaccharide changes after disappearance of polymer

Time	DP > 7	7	6	5	4	3	2	1
				Percent in oligomer				
4	35	10	20	14	13	6	1.2	0.3
9	10	3	17	23	29	15	3	0.4
30	13	1	2	11	38	24	9	1.4
72	6	1	1.5	4	35	38	12	3.5
				(percent)/DP				
4	—	1.4	3.3	2.8	3.2	2	0.6	0.3
9	—	0.4	2.8	4.6	7.2	5	1.5	0.4
30		0.1	0.3	2.2	9.5	8	4.5	1.4
72	—	0.1	0.7	0.8	8.8	12.7	6	3.5

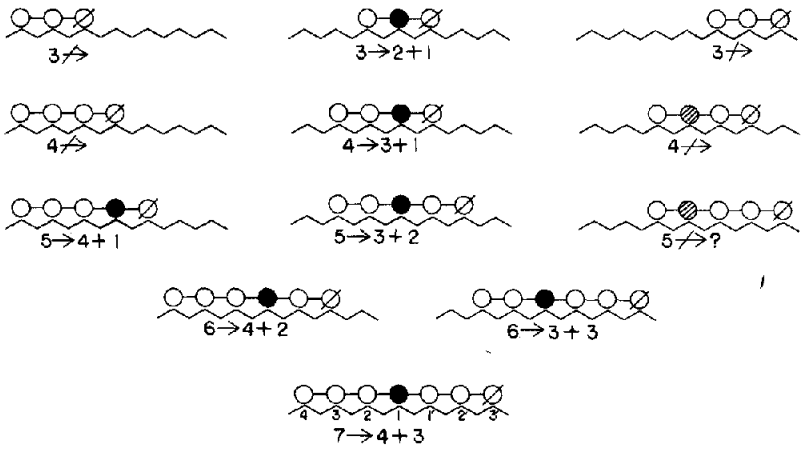

Fig. 1. Seven-subunit model of endo-polygalacturonase. The lowermost diagram shows the numbered subsites on the enzyme, represented by the zig-zag line. The oligomeric substrates are represented by the connected circles. The filled circle is the galacturonic acid unit that is activated by enzyme subsite 1, breaking the glycoside bond to its immediate right. (In non-productive modes, this is lined, not filled.) The circle with a line represents the reducing end-group. Each numerical equation represents the mode of splitting (if any) of the oligomer in question in the position shown. Two additional modes of attachment are possible for trimer, and one for tetramer. Additional modes of attachment are possible for all oligomers if some substrate units are not in contact with the enzyme. In particular, hexamer, heptamer, and larger oligomers can attach in the position shown for pentamer at the extreme left, hydrolysis releasing a monomer; similarly heptamer and larger oligomers can be positioned as the left-hand hexamer, releasing dimer. Trimer or tetramer can be released from larger oligomers by having one end or the other positioned in the same manner as heptamer. Larger oligomers are released from substrate molecules having both ends unattached to the enzyme.

endo-PG has at least 6 subsites and not 4 (see below for an alternative argument). The data from Phaff's laboratory agree, so far as they go, for the enzyme from *S. fragilis*. The data reported here extend these observations to indicate that the latter enzyme, and perhaps the former, has an active site consisting of 7 subsites. If Rexová-Benková's model of 4 subsites [1, 2] were correct, then any substrate larger than the tetramer would be hydrolysed (at comparable concentrations) at the same rate as the tetramer, which is not the case. An active site of 7 subunits is suggested rather than a larger one, since the data show heptamer beginning to disappear at the same time as large oligomers, instead of lagging behind as does hexamer.

Further results are best discussed in the context of an explicit model, presented in Fig. 1. Here, the 7 subunits are shown in two groups, numbered from the bond to be broken, subsites 1, 2, 3, 4 being toward the non-reducing end, and subsites 1′, 2′, 3′ toward the reducing end. Subsite 1 is the principal catalytic subsite, activating a galacturonic acid unit so that the glycoside bond to its right is broken. A positive free energy of attachment to subsite 1 may be assumed; the affinities of the other subsites for galacturonic acid units can be similarly represented by negative free energies of attachment, but not necessarily identical. Also shown are attached oligomers in most of the possible modes (3 of the 5 modes for trimer are shown, and 3 of the 4 for tetramer), and indications of the resulting modes of fragmentation. The trimer is shown at the left and right in 2 possible non-productive, inhibitory attachments, which are (is?) as firm as that of tetramer in the productive mode [2] shown in the middle. The hydrolysis of trimer by itself is only at 2% of the rate of tetramer by itself [5]. The tetramer attachment shown on the right does not occur to the extent of productive hydrolysis [2]. This was interpreted by Rexová-Benková [1, 2] to deny the presence of subsites 2′ and 3′, but can also be accounted for by a requirement for attachment at both subsites 2 and 3 in order to counteract the repulsion at subsite 1. If so, no other oligomer (except trimer) should be hydroylsed in this pattern to yield a dimer from the non-reducing end, and the pentamer is thus shown as non-productive. Reduced pentamer is hydrolysed to release normal trimer [1], as if it were a normal tetramer. The 4 subunit model [1, 2] accounts for the fragmentation of pentamer in two modes (3 + 2; 4 + 1) by the omission of subsites 4, 2′ and 3′, but the 7 subsite model is also capable of giving these same fragments (and further accounts for the higher rates of hydrolysis of hexamer and heptamer).

The 4 subunit model also predicts that the fragmentation of pentamer by 2 modes, and hexamer by 3 modes, are all equally probable, since no specific interactions can occur without attachment to the enzyme. Koller and Neukom [6] have reported that the preferred mode of action of their *A. niger* endo-PG on pentamer was 3 + 2, and on hexamer was 3 + 3, which is inexplicable using the 4 subunit model. The 7 subunit model (Fig. 1) requires only that subunits have different affinities for the saccharide units of the chain to account for these preferences. With another preparation [1], the pentamer was split in the 2 modes with equal probability. An attempt was made to account for the oligomer changes shown in Table 2 between 9 and 30 hr in terms of specific modes of fragmentation of the hexamer and pentamer. The monomer could only be formed by a 4 + 1 split of the pentamer, a 5 + 1 split of the hexamer followed by a 4 + 1 split of the resulting pentamer, or a similar production of monomer from larger substrates. By assuming some distribution among these modes, the other modes of fragmentation can be calculated by difference, calculating the amounts of trimer and dimer accumulating. The simplest assumption which gave reasonable results was

that monomer was derived equally from these 3 sources, an assumption in accord with the 7 subunit model. The observed changes in trimer and dimer were +9 and +6% (Table 2), while the calculated amounts were +9.7 and +7.7%. The relative probability of the different modes of fragmentation of individual oligomers, resulting from this analysis, are: pentamer is split 3 + 2 or 4 + 1 in *ca* 6:1 ratio; hexamer is split (4 + 2):(3 + 3): (5 + 1) in *ca* 10:4:1 ratio. Other assumptions as to the origin of monomer give somewhat different ratios of modes of fragmentation, but only with the improbable assumption that all the monomer resulted from the 4 + 1 split of pre-existing pentamer did the 4 + 2 and the 3 + 3 modes of hexamer hydrolysis become equal. These results differ from those cited above for enzymes from *Aspergillus* and cannot be quantitatively compared with prior work on yeast PG [5].

There is a possibility that the active site is not really as large as suggested in the model, if substrates longer than the active site were nevertheless acted on at a more rapid rate than those which are just long enough to occupy the active site. There is a higher probability that a long chain will randomly attach to the enzyme in a productive manner, than would a substrate just long enough to occupy the active site. (On the other hand, one might expect the longer chain to be acted upon more slowly, due to the slower diffusion rate of the substrate to the enzyme. This can probably be rejected as a major factor, however, because the rate of diffusion away from the enzyme would be likewise slower, leading to a greater time for action.) This statistical argument can be approached by consideration of the sizes of the fragments cut from the ends of long chains. All fragments, long enough to occupy the active site on the respective side of the broken bond, should be produced at equimolar rates, since the probabilities of a long chain randomly attaching in various configurations should be equal.

This was tested by the use of a partially degraded substrate (DP 9-50), which had been reduced with borohydride-[^{3}H], converting all the reducing end groups to labelled L-galactonic acid. In addition, the non-reducing ends had been partly converted into unsaturated groups, containing a distinctive UV absorption. This analysis was performed at pH 3.5, in contrast to the above (5.5); this may give some differences in absolute affinity [5], but the lower ionization should increase the flexibility of the substrate.

Table 3 shows the production of reduced oligomers by endo-PG at pH 3.5, during the early, linear part of the reaction. The molar amounts of pentamer, hexamer and heptamer are, within experimental error, identical at both times assayed and proportional to reaction time. In comparison to these, the average rate of production of reduced tetramer is *ca* 80%, while reduced trimer is less than 40% and reduced dimer is only 3%. The value for reduced tetramer is within the variation of the higher oligomers at 0.5 hr, but not at 0.25 hr. Yeast endo-PG is known to attack reduced oligomers as if they were actually one unit shorter [2]. This is also shown by comparison of the ^{3}H-labelled with unlabelled oligomers produced in this experiment (data not shown); labelled monomer was not produced even upon long incubation, and the early distribution of oligomers generally follows this assumption. Equating the reduced tetramer to normal trimer, etc., the minimum length of active site in the direction of the reducing end is 3 subunits. Since the fragments from the non-reducing end were all longer than trimer (below), partial confirmation of this this result could be obtained in the same experiment by examination of the normal oligomer; the early rates of production of dimer and monomer were respectively 20 and 10% of that of trimer. The 4 subunit model predicts that monomer (or reduced dimer) be produced from the reducing end at rates approaching those of higher oligomers; these data therefore support the 7 subunit model.

The overall rate of release of UV-labelled oligomers was the same as that of ^{3}H-label [7], but even during the rapid linear phase of hydrolysis, the pattern of release was very different (Table 3). The molar amounts of unsaturated pentamer, hexamer and heptamer are again almost equal, but that of the tetramer is proportionately nearly double and the amounts of trimer, dimer and monomer are all too small to measure. (Even long incubation produced very little unsaturated trimer and dimer.) If the unsaturated moiety were like the reduced moiety in not attaching to the enzyme, the length of the active site toward the reducing end would be only 3 subunits. However, on long incubation the unsaturated pentamer nearly disappears, in contrast to the reduced pentamer [7]. This and the excessive production of unsaturated tetramer (Table 3) indicate that the unsaturated moiety does interact with the enzyme and so the active site toward the non-reducing end appears to have 4 subsites as proposed (Fig. 1).

Table 3. Liberation of end groups from labelled polymer during the earliest phase

	DP of oligomer, including altered moiety						
	1	2	3	4	5	6	7
Reduced oligomer (^{3}H)							
0.25 hr	0	61	532	1100	1540	1580	1790 cpm
0.5 hr	10	70	1340	2870	3380	3360	2730 cpm
Mean/hr	—	3	37	79		100	%
Unsaturated oligomer							
0.25 hr	0	0	0	140	76	67	81 Abs. units
0.5 hr	0	0	7	281	170	152	143 Abs. units
Mean/hr	—	—	3	184		100	%

The data from the slow hydrolysis of oligomers late in the reaction at pH 5.5 are supported by the data on the rapid hydrolysis of polymer, early in the reaction at pH 3.5. The 7 subsites appear to be real.

EXPERIMENTAL

Enzyme. The culture filtrate of *S. fragilis* contains only endo-PG [8, 9]. The culture filtrate was absorbed on a preparative column of cellulose phosphate and eluted with a pH gradient [10]. The PG eluted as a single peak, corresponding to peak V of [11]. It was concentrated by ultrafiltration [12] and freeze-dried without removal of polyethylene glycol.

Gel-filtration chromatography. This has been previously described [4].

Course of hydrolysis. The data in Table 1 represent 3 separate expts, with slightly different enzyme concns (0–4 hr, 9,30 hr, 72 hr). The 9 and 30 hr times were corrected to make an earlier sample conform to the time course of the earlier series. The 72 hr time is uncorrected. All 3 were run with the same pectate sample (*ca* 1%) at pH 5.5 and 30°, using *ca* 10 mg/l. enzyme. The aliquot for viscosity was taken from the mixture after addition of enzyme and incubated in parallel. The samples for analysis were removed at the times shown and acidified with HCl, giving the ppts. shown. Reducing groups were determined by titration of alkaline hypoiodite [13].

Double-labelled pectate. A sample of pectin was partially degraded with pectin lyase, followed by alkaline saponification. The resulting pectate was reduced with NaB^3H_4 at pH 10, acidifed, and precipitated with 3 vol. of *iso*PrOH. The product had no oligomers smaller than 8–9. A detailed description of this material and the chromatography of its hydrolysis products is described elsewhere [7].

Acknowledgements—The assistance of Dorothy Gilbert Bruber and Wm. G. Nolan in enzyme preparation is acknowledged with appreciation. The culture of *S. fragilis* was gift of Dr. H. J. Phaff and maintained by Dr. W. Gauger. The substrate was a gift of Sunkist Growers, Ontario, Calif. This work was supported in part by National Science Foundation grants Nos. GB-3536 and GB-6587.

REFERENCES

1. Rexová-Benková, L. (1973) *Eur. J. Biochem.* **39**, 109.
2. Rexová-Benková, L. and Markovic, O. (1976) *Adv. Carbohydr. Chem. Biochem.* **33**, 323.
3. Dickerson, R. E. and Geis, I. (1969) *The Structure and Action of Proteins.* Harper and Row, New York.
4. McClendon, J. H. (1975) *Phytochemistry* **14**, 377.
5. Demain, A. L. and Phaff, H. J. (1954) *J. Biol. Chem.* **210**, 381.
6. Koller, A. and Neukom, H. (1968) *Eur. J. Biochem.* **7**, 485.
7. McClendon, J. H. (1979) *Plant Physiol.* (in press).
8. Phaff, H. J. and Demain, A. L. (1956) *J. Biol. Chem.* **218**, 875.
9. Phaff, H. J. (1966) *Methods Enzymol.* **8**, 636.
10. McClendon, J. H. and Kreisher, J. H. (1963) *Analyt. Biochem.* **5**, 295.
11. McClendon, J. H. and Hess, J. L. (1963) *J. Food Sci.* **28**, 289.
12. McClendon, J. H. and Somers, G. F. (1955) *Plant Physiol.* **30**, 485.
13. Jansen, E. F. and MacDonnell, L. R. (1945) *Arch. Biochem.* **8**, 97.