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## Note

# Action pattern of mannuronan C-5-epimerase: generation of block-copolymeric structures in alginates by a multiple-attack mechanism

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Mainly through the pioneering work of the late Dexter French and his collaborators[1,2], three main types of action pattern have been recognised for starch-metabolising enzymes. (*1*) *Multiple-chain*, illustrated by phosphorylase[3] and where the enzyme–substrate complex dissociates after each reaction. (*2*) *Single-chain*, exemplified by beta-amylase at its optimum pH[3] and where the enzyme–substrate complex does not dissociate until every accessible linkage in a given chain has been hydrolysed. (*3*) *Multiple attack*, typified by hog pancreatic alpha-amylase at pH 6.9, which performs an average of five hydrolytic attacks for every enzyme–substrate encounter[1]. These repeated attacks proceed towards the non-reducing end of the chain. Evidently, the multi-chain and the single-chain mechanisms are extreme examples of the more general phenomenon of multiple attack. The multiple-attack mechanism also operates with a dextranase[4] and a laminaranase[5] and is probably also responsible for the block distribution of methyl ester groups in pectinic acids, after partial de-esterification by pectinesterase[6]. However, it would be surprising if the multiple-attack mechanism were encountered only with hydrolases. Block-copolymeric structures have so far been discovered in alginates[7], complex pectins[8], heparan sulphate[9], agars and carrageenans[10,11], and in some legume-seed galactomannans[12].

We now report on strongly indicative evidence that the block structure found in many (but not all) alginates is due, at least in part, to multiple attack by mannuronan C-5-epimerase[13] upon the preformed mannuronan chain[13].

The extracellular glycuronan produced by *Azotobacter vinelandii* is a binary, linear block-copolymer of D-mannuronic acid (M) and L-guluronic acid (G)[14,15]. A distinctive feature of this polymer is the presence of *O*-acetyl groups[16], which are absent from the glycuronan of similar composition in brown, marine algae. The primary product in the bacterium appears to be a homopolymer of D-mannuronic acid that is a substrate for a mannuronan C-5-epimerase, which converts[17] in-chain D-mannuronic acid residues into L-guluronic acid residues by epimerisation at C-5. This enzyme, which is isolated from the culture media of *Azotobacter vinelandii* and purified by affinity chromatography[18], is apparently active on all unsubstituted

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blocks of two or more contigous mannuronic acid residues provided that this block forms part of a polymer of not less than 10 monomer units[19]. The enzyme requires $Ca^{2+}$ for its activity, which increases with increasing concentration of $Ca^{2+}$ until approximately equimolar amounts of $Ca^{2+}$ and substrate are present. Thus, $Ca^{2+}$ appears to be an activator in the classical sense. However, the increase in the rate of conversion effected by higher concentrations of $Ca^{2+}$ is accompanied by a change in the sequence of monomer units in the polymer produced[20], as shown in Table I. $F_M$ and $F_G$ represent the content of mannuronic and guluronic acid, and $F_{MM}$, $F_{MG}$, $F_{GM}$, and $F_{GG}$ the diad or the nearest neighbour frequencies[20] of the epimerised products. From these and other experiments, it was concluded that a low concentration of $Ca^{2+}$ favours epimerisation at positions adjacent to pre-existing G-units, thus increasing $F_{GG}$. An increase in concentration of $Ca^{2+}$ appeared to shift the epimerisation in the direction of a random process.

The diad frequencies of the product epimerised at high concentration of $Ca^{2+}$ are very close to those expected for a linear copolymer having a statistically random distribution of the two types of units along the chain (Table I). This suggested that, in this case, binding of the enzyme to the polymeric substrate occurred at random with respect to units as well as to molecules, and that the enzyme epimerised only one uronic acid residue for each enzyme–substrate association. In such a system, $Ca^{2+}$ may affect the mode of action of the enzyme, and it is suggested that a decrease in the concentration of $Ca^{2+}$ changed the action pattern from a multi-chain towards a multiple-attack process[1]. Such a change in mode of action (with pH) has been demonstrated[1] for hog pancreatic alpha-amylase. Since we are dealing with an enzyme that changes only the identity of individual units in a pre-existing polymeric chain, a change from a multi-chain to a multiple-attack mechanism will have a pronounced effect on the distribution of the epimerised units. The effect on the molecular level will be to increase substantially the occurrence of reacted diads ($F_{GG}$), as amply demonstrated in Table I. However, the distribution of reacted units among molecules will also be influenced by such a change. This is most easily appreciated by envisaging an increase in the degree of multiple-attack (the number of units transformed per productive enzyme–substrate association). Evidently the multiple-attack process then approaches a single-chain mechanism. With an

TABLE I

COMPOSITION AND DIAD FREQUENCIES[20] OF PRODUCTS OF THE EPIMERASE REACTION[a] AT LOW AND HIGH LEVELS OF $Ca^{2+}$ IONS

| $Ca^{2+}$ (mM) | $F_M$ | $F_G$ | $F_{MM}$ | $F_{MG}$ | $F_{GM}$ | $F_{GG}$ |
|---|---|---|---|---|---|---|
| 0.85 | 0.62 | 0.38 | 0.51 | 0.11 | 0.11 | 0.27 |
| 3.40 | 0.69 | 0.31 | 0.46 | 0.23 | 0.23 | 0.08 |
| Random distribution | 0.69 | 0.31 | 0.48 | 0.21 | 0.21 | 0.10 |

[a]Initial mannuronan concentration, 3.7mM.

TABLE II

FRACTIONATION OF EPIMERISED ALGINATE WITH $Ca^{2+}$ AND COMPOSITION OF THE FRACTIONS

| | *Yield (mg)* | $F_G$ | $F_M$ | $F_{MM}$ | $F_{MG}$ | $F_{GG}$ |
|---|---|---|---|---|---|---|
| Soluble | 45 3 | 0.08 | 0.92 | 0 86 | 0.06 | 0.02 |
| Insoluble | 9 8 | 0.43 | 0.57 | 0 40 | 0.17 | 0 26 |

epimerase working according to the latter mechanism, the composition–distribution curve will always be bimodal, at least at moderate degrees of conversion; consequently, the system will be compositionally heterogeneous. Therefore, the degree of heterogenity will depend on the degree of multiple attack. A theoretical study of this dependence will be published elsewhere.

The variation in physical properties of the alginate with uronic-acid composition has been utilised to demonstrate that the population of molecules following epimerisation with mannuronan C-5-epimerase is compositionally heterogeneous. The results are given in Table II.

A polymeric substrate (d.p. >50 containing >95% of mannuronic acid) was incubated[21] for 2 h at 30° with a sample of affinity-purified enzyme[21] in the presence of 0.68mM $Ca^{2+}$. The reaction was stopped by addition of EDTA (50mM), and the sample was dialysed against distilled water, the alginate concentration was adjusted to 5 mg/mL, and aqueous calcium chloride was added to a final concentration of 8mM. The precipitate and supernatant fractions were separated by centrifugation, freed from $Ca^{2+}$ ions by dialysis, and analysed for total uronic-acid content by the phenol–sulphuric acid method[22]. They were then freeze-dried, and solutions of the residues in $D_2O$ were analysed for uronic acid composition and sequence by 400-MHz $^1$H-n.m.r. spectroscopy[23]. The results are shown in Table II. Because of the imperfection in the fractionation procedure, it is not possible to attribute any significance to the small proportion (8%) of guluronic acid residues in the soluble fraction. Virtually all the epimerisation had occurred in the insoluble fraction, which represented 18% of the total material containing 48% of the guluronic acid. The results clearly demonstrate compositional heterogeneity in the molecular population, and this is a strong indication of a multiple-attack mechanism as the most probable mode of action of the C-5-epimerase. It is clear that the block-copolymeric structure of the alginate synthesised by *Azotobacter vinelandii* is due, at least in part, to multiple attack by the C-5-epimerase on a preformed mannuronan chain. This may not be the only explanation, however, because the bacterial polymer contains *O*-acetyl groups, which also influence the action pattern of the enzyme[24]. Most alginates from brown algae also have block structures, but there is no evidence for the introduction of *O*-acetyl groups at any stage in their biosynthesis. The present results provide an indirect experimental basis for believing that these materials also owe their block structure to a multiple-attack mechanism.

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