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The fine structure of oyster glycogen*

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ABSTRACT

When oyster glycogen was hydrolysed in sequence with beta-amylase, isoamylase, and beta-amylase, fractionation of the products on Bio-Gel P2 revealed that the smallest and most abundant maltosyl-Ba chain fragment had d.p. 5, and carried 1 maltosyl residue. A considerable amount of maltotriose was produced from Ba chains by the debranching of maltosyl-Ba chain fragments. The Ba chains carried increased numbers of A chains with increase of the chain length. In the outermost layer of the molecule, there were abundant 1–2 intervening (1→4) linkages for an A chain bound to a Ba chain. High performance anion-exchange chromatography with pulsed amperometric detection showed that the individual chains of the glycogen debranched with isoamylase were in the range from d.p. 2 to 30 and that the chain with d.p. 6 was the most abundant. The results are compatible with the random branching structure.

INTRODUCTION

Glycogen and amylopectin comprise (1→4)-α-D-glucan chains with branches that involve α-(1→6) linkages. These highly branched structures are fully or partially degraded by debranching enzymes[1–17] into (1→4)-α-D-glucans of various lengths. Many attempts have been made to analyse the fine structures of glycogen and amylopectin, but, although several models[7,14–21] have been proposed, the fine structures are not yet fully understood[17].

Peat *et al.*[22] classified chains as A chains (each linked to another chain solely through its reducing residue), B chains (each has branch linkages at C-6 and at its reducing residue), and C chains (each has free reducing residues). The ratio of A and B chains has been proposed for the characterisation and analysis of these polysaccharides[17,22–27]. Recently, Hizukuri and Maehara[28] classified the B chain into Ba and Bb chains; the former carry at least one A chain and the latter carry no A chain but B chains. A new enzymic method was reported for the analysis of the number of A chains bound to a B chain, and thus the structure of wheat amylopectin was characterised in detail.

Glycogen has more branches than amylopectin and a significantly different chain-length (c.l.) distribution[7]. Glycogen and amylopectin show monomodal[9,10,15] and

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polymodal[20] c.l. distributions, respectively. We now report elucidation of the fine structure of glycogen by applying the new enzymic technique[28].

EXPERIMENTAL

Materials. — Oyster glycogen Type II (Sigma) was purified by precipitation with ethanol (1 vol.) from a 2% solution in distilled water. The process was repeated two more times. The beta-amylolysis limit was 42% and the average chain length ($\overline{c.l.}_n$) was 11. Crystalline *Pseudomonas* isoamylase and *Klebsiella* pullulanase were obtained from Hayashibara Biochemical Laboratories (Okayama). Twice-crystallised beta-amylase was prepared[30] from sweet potato and crystallised again from aqueous ammonium sulfate (0.36–0.40 saturation, pH 3.7). Other reagents were of the highest grade commercially available.

Preparation of beta-amylase limit dextrin (β-LD). — Oyster glycogen (4 g) was incubated with crystalline sweet-potato beta-amylase (4×10^4 U) in 50mM acetate buffer (200 mL, pH 4.8) in a cellulose tube (Visking Co.) at 37° for 48 h, with dialysis against the same buffer which was renewed eight times, four of which were during the first 12 h. The reaction was terminated by heating the mixture for 10 min in a boiling water bath. The coagulated protein was removed by centrifugation, and the supernatant solution was mixed with ethanol (200 mL) and aqueous 0.1% lithium bromide (40 μL), then centrifuged. The resulting precipitate was dissolved in water (80 mL) and reprecipitated by adding ethanol (80 mL) and aqueous 0.1% lithium bromide (40 μL). This step was repeated twice, and the β-LD (1.9 g) recovered by centrifugation was washed by suspension in ethanol and then ether, and dried under reduced pressure over calcium chloride in a desiccator.

Preparation of maltosyl (G_2)-Ba chain fragments. — A solution of β-LD (500 mg) in 5mM acetate buffer (100 mL, pH 3.5) was treated with isoamylase (75 U) at 45° for 5 h. The reaction was terminated by heating in a boiling water bath for 15 min. The pH was adjusted to 4.8 with M sodium hydroxide, beta-amylase (10 U/mg of β-LD) was added, and the mixture was incubated at 37° overnight (16 h). The reaction was stopped by boiling the mixture for 15 min, the mixture was then filtered through a G4 filter and freeze-dried, and the residue was eluted from a column (3.2 × 90 cm) of Bio-Gel P-2 with water at a flow rate of 25 mL/h (5-mL fractions). Glucose, maltose, and maltotriose were removed, and the remaining products were combined, concentrated, and treated again at 37° overnight with an excess of beta-amylase (50 U/mg) after adjusting the pH to 4.8. The reaction was stopped by heating in a boiling water bath for 15 min, and the solution was filtered through a G4 filter and freeze-dried to give the G_2-Ba chain fragments (120 mg). These fragments were again subjected to chromatography on Bio-Gel P-2 and divided into fractions (A–D) based on equal carbohydrate content (Fig. 1).

Preparation of Ba chain fragments. — G_2-Ba chain fragments (1 mg/mL) were debranched with pullulanase (2.5 U/mg) at pH 6.0 for 6 h at 40°, and the reaction was stopped by boiling for 15 min. The resulting protein precipitate was removed by centrifugation.

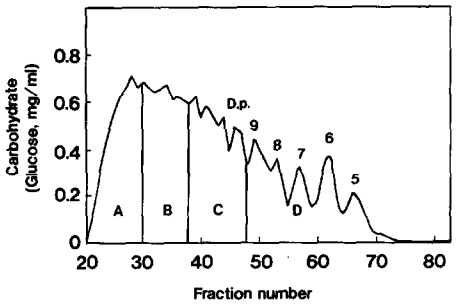

Fig. 1. Fractionation of G_2-Ba chain fragments on Bio-Gel P2.

Preparation of debranched oyster glycogen. — Oyster glycogen (100 mg) was dissolved in water (5 mL) at ~ 100°. The solution was cooled, M acetate buffer (100 μL, pH 3.5) was added, and the pH was adjusted to 3.5 with 0.1M HCl. Isoamylase (30 U) was added and the mixture was incubated for 12 h at 45°. The reaction was terminated by boiling for 10 min and the mixture was lyophilised.

A portion (~ 1.5 mg) of the residue was dissolved in M sodium hydroxide (200 μL), the volume was made up to 1 mL, the solution was filtered through a 0.22-μm filter (Millex GS, Millipore Corp.), and an aliquot (25 μL) was used for high-performance anion-exchange chromatography[31] (h.p.a.e.c.).

General analytical methods. — Total carbohydrate was determined by the phenol–sulphuric acid method[32], and reducing residues by the method of Somogyi[33] and Nelson[34] with extended heating for 30 min. Non-reducing residues were determined by enzymic assay of glycerol after periodate oxidation and reduction with sodium borohydride[35]. The assays of the chain length and beta-amylolysis limit were performed as reported[36]. The number-average degree of polymerisation ($\overline{d.p._n}$) and number-average chain length ($\overline{c.l._n}$) were calculated on the basis of the proportions of reducing and non-reducing residues, respectively. One U of isoamylase is defined as the amount of the enzyme which produces 1 μmol of reducing power as glucose per min from soluble waxy-rice starch at 45° and pH 3.5.

Debranching of β-LD with isoamylase. — The pH of an aqueous solution of β-LD (0.5–1.0 mg/5 mL) was adjusted to 3.5 with M HCl, isoamylase (0.04–20 U/mg) was added, and the mixture was incubated at 45°. Aliquots were removed at intervals, the reaction was stopped by adding 150mM sodium hydroxide, and the samples were stored at ~ 50°, filtered through a 0.22-μm membrane filter, and analysed by h.p.a.e.c.

Determination of the A:B chain ratio. — The pH of a solution of β-LD (5 mg) in water (5 mL) was adjusted to 3.5 with M HCl, isoamylase (0.3 U/mg of β-LD) was added, and the mixture was incubated at 45° for 8 h. The reaction was stopped by boiling for 15 min. The pH was adjusted to 6.0 with M sodium hydroxide, pullulanase (2.5 U/mg of β-LD) was added, and the mixture was incubated at 40° for 4 h. The reaction was stopped by boiling for 15 min. The total amount (mol) of reducing sugar was determined[33,34]. The remaining hydrolysate was frozen at − 10°, then thawed at room

temperature, and filtered through a membrane filter (0.22 μm, Millipore Corp.). The total maltose and maltotriose was determined by using h.p.a.e.c. The A:B chain ratio = $(G_2 + G_3)/(\text{total} - G_2 - G_3)$.

High-performance anion-exchange chromatography (h.p.a.e.c.). — A Dionex BioLC model 4000i system was used with a Model PAD II pulsed amperometric detector, an amperometric flow-through cell with a gold working electrode, a potentiostat, and a Dionex CarboPac PAI (250 × 4 mm) column with an AG6 guard column (50 × 4 mm). The operating pulsed potentials (V) and durations (ms) were as follows at range 2 (sampling period, 200 ms): E_1 0.10 (t_1 300); E_2 0.60 (t_2 120); E_3 0.80 (t_3 300). The response time of the PAD II detector was set to 1.0 s. Eluent A was 150mM sodium hydroxide prepared from carbonate-free aqueous 50% sodium hydroxide in 18 MΩ.cm deionised and distilled water. Eluent B was 150mM sodium hydroxide containing 500mM sodium acetate.

The eluents selected were for (*a*) assay of the maltose and maltotriose, and determination of the A:B chain ratio: eluent B = 20% and eluent A = 80%; (*b*) for analysis of chain-length distribution in debranched glycogen, maltosyl-Ba chain fragments, and Ba chain (of the whole fragment): eluent B = 40% at 0 min, 50% at 10 min, 60% at 25 min, and 70% at 40 min; and (*c*) for assay of the G_2-Ba and Ba chain fragments D: eluent B was 30% at 0 min and 40% at 15 min; 10–25-μL aliquots of the specimens were used.

RESULTS AND DISCUSSION

The oyster glycogen had a beta-amylolysis limit (42%) and $\overline{\text{c.l.}}_n$ (11.0) similar to those reported (39–44%[7,17,26,39] and 10–12[13–17,37–42], respectively). The apparent ratio of A and B chains was 1.24:1, as calculated from the amounts of G_2 and G_3 produced after complete debranching of the β-LD. As discussed below, this value was inaccurate. The c.l.-distribution profile, determined by h.p.a.e.c. (Fig. 2) revealed quantitatively the presence of small proportions of G_2–G_5 chains and a series of chains with d.p. up to ~ 30. Fig. 3 shows the relative molar distribution of chains up to d.p. 17, which was calibrated on the basis of the molar responses[31]. The chain with d.p. 6 was by far the most abundant. Chains with d.p. >7 gradually decreased in a hyperbolic curve, as reported by Palmer *et al.*[42]. From the average external c.l. of 6.6, the chains with d.p. <7 are possibly all A chains. This distribution profile differed from those of the amylopectins[31], for which the most abundant chains had d.p. 10–13.

The mode of connection of the A and Ba chains was analysed through the structure of the G_2-Ba chain fragments, which were the products of the degradation of the glycogen in sequence with beta-amylase, isoamylase, and beta-amylase ($\beta \cdot i \cdot \beta$-analysis). The key to the preparation is the controlled hydrolysis of β-LD with isoamylase to reduce the liberation of maltose. The ideal conditions are complete hydrolysis of all branch linkages except those linking maltose. However, isoamylase can slowly hydrolyse the G_2-branch linkage. The liberation of maltotriose from β-LD with the enzyme (0.15 U/mg of substrate) attained a maximum at 4 h, whereas that of maltose

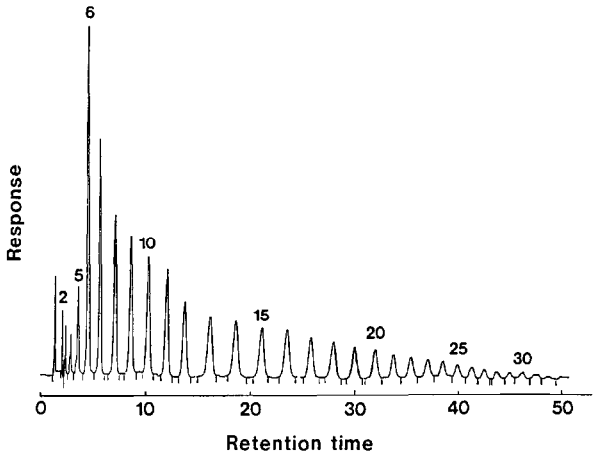

Fig. 2. C.l. distribution of debranched oyster glycogen by h.p.a.e.c.; the numbers indicate the d.p.

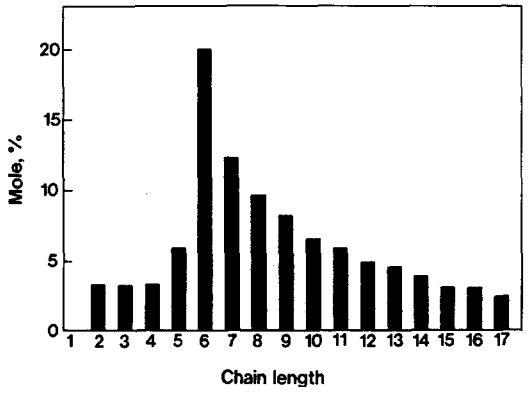

Fig. 3. Calibrated c.l. distribution of oyster glycogen.

continued to increase gradually. Since the liberation of $<20\%$ of maltose does not cause significant error in this analysis[28], the hydrolysis was terminated after 5 h, when 10.2% of the maltose had been liberated and all other branch linkages hydrolysed. The maximum level of the maltotriose was attained within 30 min with a higher amount of the enzyme (20 U/mg β-LD) and there was no more hydrolysis up to 8 h. The liberation of maltose was also completed with the high amount of the enzyme (20 U/mg) at 6 h. Therefore, full debranching is possible by using the enzyme alone.

The G_2-Ba chain fragments were fractionated into 4 nearly equal parts by weight (Fig. 1) and the structural properties were summarised in Table I. The R value, the ratio of non-reducing to reducing residues, increased from 2.0 to 2.9 with increase of molecular size. These R values imply that the G_2-Ba chain fragments carry 1–1.9 maltose stubs. The $\overline{c.l.}_n$ of the Ba chain fragment was calculated by the subtraction of

TABLE I

Properties of maltosyl-Ba chain fragments of oyster glycogen.

Property	*Fraction*				
	A	*B*	*C*	*D*	*Whole*
$\overline{D.p._n}$ of G_2-Ba chain fragment	20.9	14.5	10.0	6.2	10.3
R[a]	2.9	2.5	2.3	2.0	2.2
Number of G_2 stub	1.9	1.5	1.3	1.0	1.2
$\overline{C.l._n}$ of Ba chain fragment	17.1	11.5	7.4	4.2	7.9
N[b]	3.4	2.4	1.9	1.0	1.6
G_2-Ba chain (%)					
In mole	13	18	26	43	100
By weight	25	26	24	25	100

[a] Non-reducing residue:reducing residue. [b] Number of A chains binding to a Ba chain in glycogen.

twice the number of G_2 stubs from the $\overline{d.p._n}$ of each fraction. The $\overline{c.l._n}$ of the Ba chain fragments, thus obtained, ranged from 4.2 to 17.1. On the other hand, the *N* value, the number of A chains linked to a Ba chain in native glycogen, was calculated from the *R* value by eqn. *1* where *C* means combinations[28].

$$R = \sum_{i=1}^{N} {}_NC_i\,(1 + i)/(2^N - 1) \qquad (1)$$

Equation *1* is based on the following assumptions: (*a*) all of the A chains are degraded to maltose or maltotriose by beta-amylase, (*b*) maltose and maltotriose are derived only from A-chain stubs and these amounts are equal, and (*c*) liberation of maltose with isoamylase is $<20\%$ of the total maltosyl stubs. Assumptions (*a*) and (*c*) depend on the experimental conditions, whereas (*b*) varies with the properties of the specimen.

Assumption (*b*) was found to be correct for wheat amylopectin[28]. However, this assumption was not true for oyster glycogen, because the liberation of maltose and maltotriose from the β-LD by complete debranching was in the ratio 1.2:1, and a considerable amount of maltotriose also was derived from Ba chains (see below). In fact, the ratio of the chains with even-numbered and odd-numbered glucosyl residues between c.l. 2–17 (Fig. 3) was 1.25:1, although the ratio for the longer chains was probably 1:1, judging from the distribution (Fig. 2). Thus, the real ratio of maltosyl and maltotriosyl stubs of the A chains should be much larger than 1.2:1; therefore, the relationship between *R* and *N* expressed by eqn. *1* is not strictly valid. The *N* values in Table I indicate maximum values, and the real *N* values are considered to be intermediate between $R - 1$ and *N*. However, the smallest Ba chain fraction, $\overline{c.l._n}$ 4.2, carried one A chain. The largest Ba-chain fraction ($\overline{c.l._n}$ 17.1) bound at most 3.4 A chains based on the *N* value, which implies that it carries 3 or 4 A chains, but most of the chains would probably carry 2 or 3 chains, and the intermediate Ba-chain fractions, C and D appeared

to be linked to 1–3 A chains. The $\overline{c.l.}_n$ of the whole Ba-chain was 7.9 and, in native glycogen, had on average at most 1.6 A chains. This value is significantly lower than that for amylopectin[28]. However, the trend that the long Ba chain carries an increased number of the A chains was similar to that of wheat amylopectin. This mode of A-chain distribution does not fit the regular branching model[7], in which the outer B chains carry 2 A chains, but it is compatible with the irregular branching model[18]. A Ba chain of glycogen carries fewer A chains than amylopectin that is less branched than glycogen, and the $\overline{c.l.}_n$ of the Ba chain fragments of glycogen (7.9) are much shorter than those (15.3) of wheat amylopectin[28].

The G_2-Ba chain fragments were subjected to h.p.a.e.c. (Fig. 4). Fraction D revealed the major peaks (N') of 5′–9′ and several minor peaks (N' indicates the d.p. of the respective component). These major peaks had retention times between those of (1→4)-α-glucan chains with d.p. $N-1$ and N, and were converted into malto-oligosaccharides (with d.p. 3–7) and maltose by debranching with pullulanase (Fig. 5). Some minor peaks, eluted faster than 5′ and between the main peaks, were not identified. Since (1→6)-α-glucans are eluted faster than (1→4)-α-glucans[43], the elution behavior of the individual component of the G_2-Ba chain fragments (5′–9′) is reasonable. Since a considerable amount of maltotriose was produced by debranching from the Ba-chain fragments in addition to A-chain stubs of the β-LD, the calculated value of the A:B chain ratio (see above) is too high. Umeki and Yamamoto[37] have used a similar argument based on the difference of the hydrolysis of oyster glycogen β-LD with pullulanase and isoamylase. They (*a*) suggested that pullulanase preferentially debranches the A chains and also the short B chains of the β-LD by the liberation of maltotetraose–maltoheptaose with pullulanase, and that 66% of the total liberated maltotriose by complete debranching belongs to the A chains; and (*b*) reported the uneven production of maltose (G_2) and maltotriose (G_3) (G_2/G_3 = 1.29) from the β-LD by complete debranching. The value agrees with our findings (1.2).

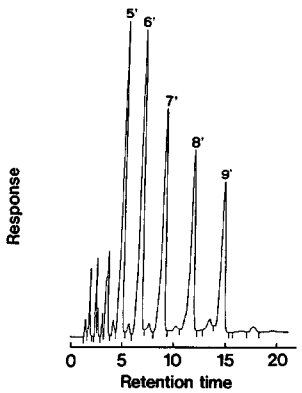

Fig. 4. H.p.a.e.c. components of G_2-Ba chain components D; the numbers indicate the d.p.

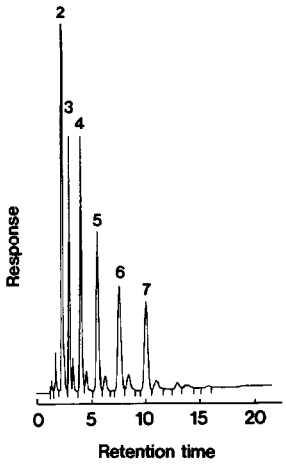

Fig. 5. H.p.a.e.c. of the hydrolysate of G_2-Ba chain fragments D with pullulanase; numbers indicate the d.p. of the malto-oligosaccharides.

Fig. 6 shows the h.p.a.e.c. elution profile of whole G_2-Ba chain fragments. A series of main peaks (denoted by N'), with retention times between these of the linear chains of d.p. $N-1$ and N, appear to be d.p. N chains having one maltosyl branch as discussed above. The minor chains (denoted by N'' in Fig. 6) located between $N'-1$ and N', may be the d.p. N chains carrying two maltosyl branches, although they were not identified. Since the G_2-Ba chain fragments were no longer hydrolysed with beta-amylase, the N'' series, which had retention times close to those of the linear series, could not be linear. The chain-length-distribution chromatogram of the Ba chain fragments, which were debranched products of the G_2-Ba chain fragments with pullulanase (Fig. 7), revealed long chains up to d.p. 30, as long as those of the native glycogen. These results suggest the presence of such long Ba chains carrying 5 or 6 A chains. The calibrated distribution of the Ba chain fragments of d.p. 3–17 showed that G_3 was the most abundant and that

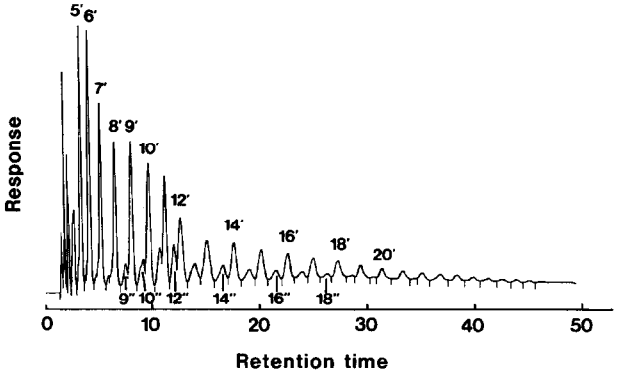

Fig. 6. H.p.a.e.c. of the G_2-Ba chain fragments (for identification of N' and N'', see text).

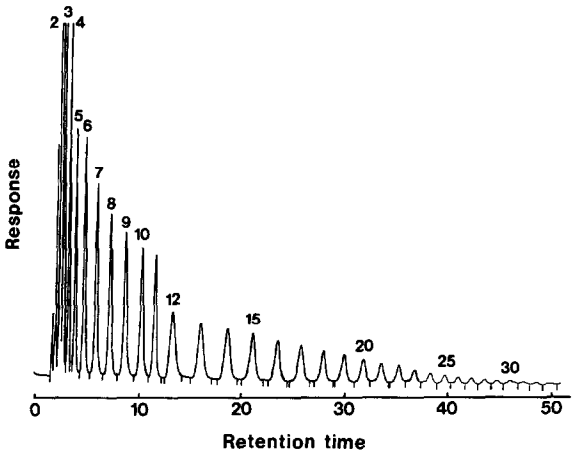

Fig. 7. H.p.a.e.c. of the hydrolysate of the G_2-Ba chain fragments with pullulanase; numbers indicate the d.p.

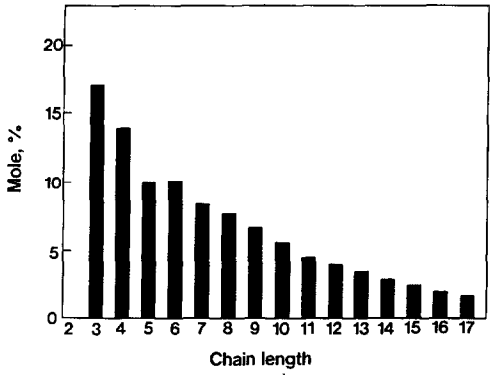

Fig. 8. Calibrated c.l.-distribution of the Ba chain fragments.

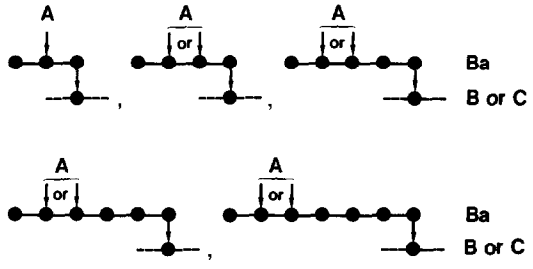

Fig. 9. Close branch points between the A and the Ba chains of the fraction D: ●, Glc; ——, α-(1→4) linkage; ↓, (1→6) linkage; ---, (1→4)-glucan chain.

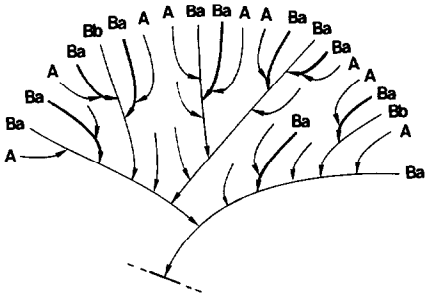

Fig. 10. A hypothetical structure for glycogen: ——, (1→4)-glucan chains; ——, Ba chain in the laminated structure (see text); ↓, α-(1→6) linkage.

the longer chains decreased hyperbolically (Fig. 8). This abundant formation of maltotriose is characteristic for the glycogen because amylopectin yields only a small amount[28]. Fig. 9 shows the close locations of two branch linkages [intervending (1→4) linkage] in the glycogen, based on the structures of the Ba chains in fraction D. A model for these A and Ba chains, together with a small number of other B (or C) chains, is shown in Fig. 10. These structures are thought to be located mostly in the outermost layer of the β-LD, and are probably more abundant in native glycogen than in G_2-maltosyl fragments because maltotriosyl stubs are removed by the isoamylolysis, which reduces the relative yield of the fraction. From the biosynthesis viewpoint, some A and Ba chains are linked so closely as to be separated by only one or two α-(1→4) linkages.

The numerical ratio of G_2 and G_3 production from β-LD by debranching was higher than the value (1:0.98) reported by Bathgate and Manners[26], but seems to be reasonable from the fact that the G_6 chain was by far the most abundant. The A:B chain ratio could be determined also from (*a*) the difference of the reducing values obtained when β-LD was degraded by the joint action of isoamylase and pullulanase and by the sole action of isoamylase[27], and (*b*) the amounts of G_2 and G_3 obtained from β-LD by the combined action of beta-amylase and pullulanase[44]. These methods are based on the assumptions that isoamylase does not act on G_2 stubs and that the amounts of G_2 and G_3 stubs of β-LD are the same, and that half of the G_3 is derived from A and B chains. The former method is not sound because it is difficult to find the conditions under which isoamylase hydrolyses all of the branch linkages but none of the G_2 stubs. More G_2 was produced than G_3 from the β-LD by debranching, so that the latter method is also not applicable and the true A:B ratio remains undetermined. Judging from the average A:Ba value (1.6) of oyster glycogen and that (2.1) of wheat amylopectin, for which the A:B ratio is 1.26:1 (ref. 28), the A:B value of the oyster glycogen would be much less than the apparent value of 1.24:1.

The β·i·β-analysis of oyster glycogen suggests that up to 4 A chains may be linked to a Ba chain, *i.e.*, the glycogen comprises at least 4 tiers (because some B chains

may be linked to the Ba chain). This view agrees with the findings of previous workers, using beta-amylase and pullulanase stepwise[14], and the classical method of sequential degradation with phosphorylase and amylo-1,6-glucosidase[19].

The structure of glycogen depends on the nutritional conditions as reported[45] in *Saccharomyces cerevisiae.* Therefore, the present analytical data on oyster glycogen may not be typical. However, since the commercial specimen is prepared from many fresh oysters, the structure may be average. It is concluded that the random branched-structure[18] is the most reasonable model for the oyster glycogen. Fig. 10 also shows the presence in the macromolecule of numerous elements of small numbers of chains arranged in a laminated pattern, resembling in this respect the structure originally proposed by Haworth[46].

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