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Note

Location of phosphate groups in potato amylopectin*

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Potato starch contains a small proportion of covalently bound phosphate in its amylopectin component[1–4], and the phosphate groups are located at C-6[4–6] and C-3[7] of the glucosyl residues. Digestion of the starch and amylopectin with amylases produces phosphorylated dextrins or oligosaccharides[6–10]. Tabata *et al.*[11] obtained phosphorylated, linear (1→4)-α-D- glucans with a $\overline{\text{d.p.}}$ of 6.25 by the hydrolysis of the starch with *B. subtilis* alpha-amylase, suggesting that no (1→6)-α linkages are present near the phosphate group. However, the location of the phosphate groups in the amylopectin is unknown. We now report that the phosphate groups are located mostly in B-chains[12] and are distributed over the whole chains except in the vicinity of the branch linkages.

The potato amylopectin used in this study contained one phosphate group per 317 glucosyl residues, that is, one in 13 unit chains of the amylopectin (average chain-length of 24) carried a phosphate group, assuming that the groups were distributed evenly on the chain. This minor, phosphorylated unit-chain (PUC) was collected by ion-exchange chromatography after debranching of the amylopectin with *Pseudomonas* isoamylase[13], as described previously[10]. PUC is a linear molecule with a $\overline{\text{d.p.}}$ of 42 bearing approximately one phosphate group, and has no phosphate attached to the non-reducing residue[10]. It was found that the phosphate group was attached mainly to the larger unit-chains of the amylopectin, because the average chain-length of PUC was considerably larger than that ($\overline{\text{d.p.}}$, 24) of the whole amylopectin. PUC was fractionated into F_1 (89%) and F_2 (11%), with $\overline{\text{d.p.}}$ values of 80–28 ($\overline{\text{d.p.}}$, 56 at the peak) and 28–20 ($\overline{\text{d.p.}}$, 22 at the peak), respectively (Fig. 1). The beta-amylolysis limits of PUC and the fractionated PUC on Bio-Gel P-60 filtration were 54 and 43–59%, respectively (Fig. 1). The molecular size of beta-limit PUC, which was fractionated by Bio-Gel P-30, was distributed widely with $\overline{\text{d.p.}}$ of 80–10 (with a peak at $\overline{\text{d.p.}}$ 34); no molecules with $\overline{\text{d.p.}}$ <10 were found (Fig. 2). These results suggest that the phosphate group is located statistically near the centre of the unit chain and more

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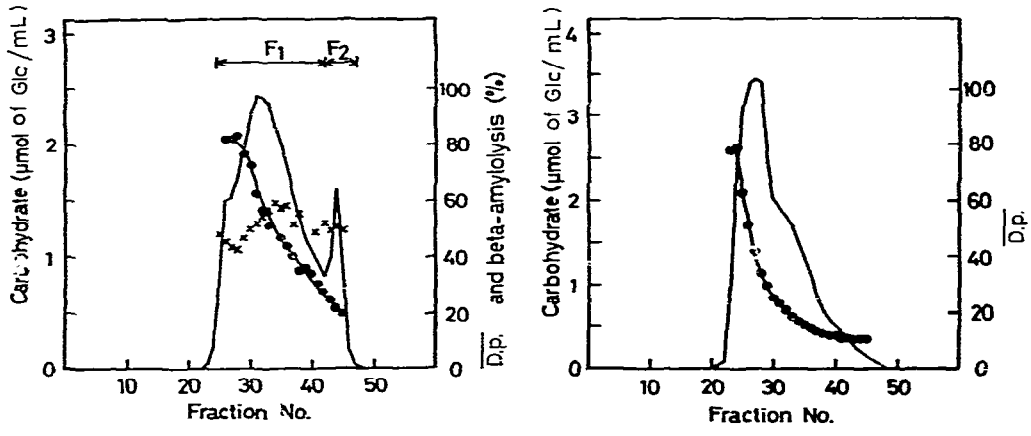

Fig. 1. Elution pattern of phosphorylated unit-chain (PUC) on Bio-Gel P-60. PUC (26.8 mg, 2.1 mL) was applied on a column (2.6 × 100 cm, packed with Bio-Gel P-60), which was maintained at 50° and eluted upwards with water. Fractions of 5 mL were collected. Beta-amylolysis (x) was carried out at 37° for 2 h in 50mM acetate buffer (pH 4.8) with 43 units of beta-amylase per mL of the fraction. ——, Carbohydrate; ●, $\overline{d.p.}$

Fig. 2. Elution pattern of beta-limit, phosphorylated unit-chain (beta-limit PUC) on Bio-Gel P-30. Beta-limit PUC, which was prepared by the digestion of PUC [80 mg in 8 mL of 25mM acetate buffer (pH 4.8)] with beta-amylase (10^4 units) for 4 h at 37°, was applied on a column (2.6 × 100 cm) packed with Bio-Gel P-30. Other conditions were as noted in Fig. 1. Maltose was eluted near fraction 110. ——, Carbohydrate; ●, d.p.

than nine glucosyl residues separated from the reducing end (*i.e.*, from a branch point), since beta-amylase hydrolyses PUC until no or one glucosyl residue remains attached to the 6-phosphorylated glucosyl residue[10]. This finding is inconsistent with the suggestion of Rodomski *et al.*[14] that the phosphate group is located close to the branch point, but is consistent with the linear structure of the phosphodextrin obtained by the hydrolysis of potato starch with *B. subtilis* alpha-amylase[11].

To identify the phosphorylated B-chains, the beta-limit dextrin of the amylopectin (beta-amylolysis limit, 56%) was debranched with *Pseudomonas* isoamylase, and only the phosphorylated chains (iβL-PD) were collected by an ion-exchange resin[10]. By these treatments, the phosphorylated B-chains are changed into linear and branched chains carrying one or more maltosyl stubs, since beta-amylase trims the A-chains to maltosyl or maltotriosyl stubs[15] and bacterial isoamylases remove the maltotriosyl stub, but not the maltosyl stub[16–19]. We found that iβL-PD had about one phosphate (1.1 mol per chain) and 1.45 mol of the non-reducing residue per chain with a $\overline{d.p.}$ of 29. It appears to be debranched completely with pullulanase and produced 0.42 mol of reducing sugar per chain. Almost all (94%) of the liberated reducing-sugar was shown to be maltose with maltose phosphorylase. No molecule of iβL-PD appears to have more than two branches, since the $\overline{d.p.}$ of each fraction of iβL-PD fractionated by Bio-Gel P-30 did not decrease by less than a half on debranching with pullulanase (Fig. 3). These results suggest that iβL-PD is composed of linear and singly branched molecules with a maltosyl stub, and that the molar

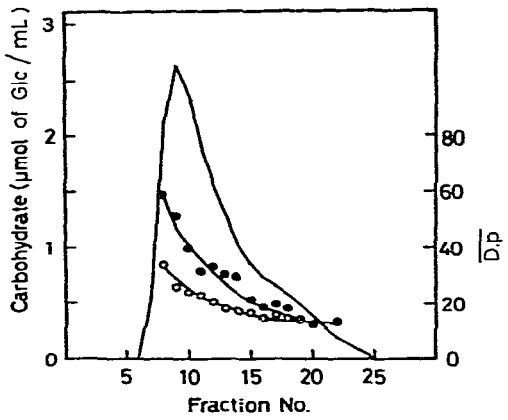

Fig. 3. Fractionation of isoamylase-debranched, beta-limit phosphodextrin (iβL-PD) on Bio-Gel P-30. iβL-PD (13.1 mg, 2 mL) was applied on a column (1.9 × 45 cm) containing Bio-Gel P-30. Fractions of 4 mL were collected. Other conditions were as noted in Fig. 1. Each fraction of iβL-PD was debranched with pullulanase (1.1 unit/mL) at 30°, for 6 h. ——, Carbohydrate; ●, $\overline{d.p.}$; ○, $\overline{d.p.}$ after pullulanase treatment.

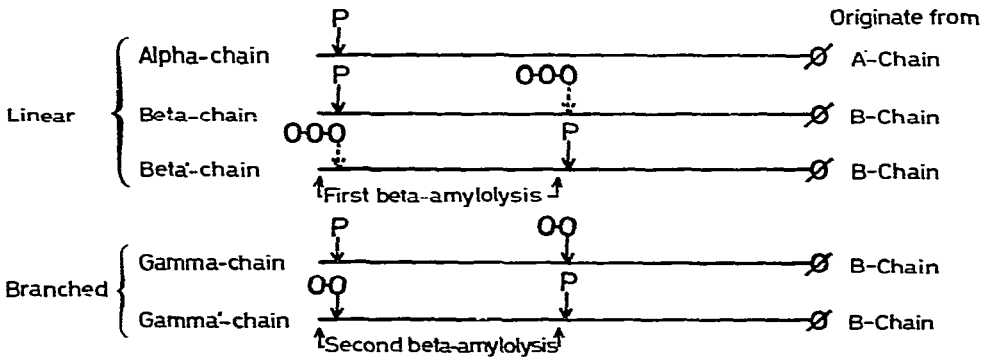

Fig. 4. Possible structures of iβL-PD. Key: ——, (1→4)-linked α-D-glucosyl residues; P, phosphate group bound to a glucosyl residue; O-O, maltosyl stub; ↓first beta-amylolysis↓, glucosyl residues removed by first beta-amylolysis; ↓second beta-amylolysis↓, glucosyl residues removed by second beta-amylolysis after the treatment with pullulanase; O-O-O, maltotriosyl stub presented in beta-limit dextrin and removed following debranching with the isoamylase.

fractions of the linear and branched molecules in iβL-PD were 0.56 and 0.44 (average value of the non-reducing residue and the reducing residue produced with pullulanase), respectively.

Among the five possible chains of iβL-PD (Fig. 4), only the alpha-chain originates from the A-chain, and all of the remainder originate from the B-chain. The linear beta- and beta'-chains result from removal of a maltotriosyl stub of the B-chain when beta-limit dextrin is debranched with the isoamylase. Accordingly, the molar

TABLE I

HYDROLYSES[a] OF THE ISOAMYLASE-DEBRANCHED, BETA-LIMIT PHOSPHODEXTRIN (iβL-PD) WITH BETA-AMYLASE AND PULLULANASE

Order of hydrolyses with enzymes	*Overall degradation of iβL-PD (%)*	*Overall degradation with beta-amylase (%)*
Beta-amylase (first)	10	10
Beta-amylase (first)→pullulanase	13	—
Beta-amylase (first)→pullulanase→beta-amylase (second)	21	18

[a] iβL-PD was incubated in 20mM acetate buffer (pH 5.5) with 12.5 units of beta-amylase per μmol of the substrate (as glucose) for 2 h at 37°. After termination of the digestion by heating, 0.5 unit of pullulanase per μmol of the substrate (as glucose) was added and the mixture was incubated at 30° for 6 h. After heating the mixture, the second digestion with beta-amylase was done under the same conditions as for the first digestion.

fraction of the B-chain is twice as much as that (0.44) of the branched chains (gamma- and gamma'-chains), assuming that the numbers of the maltosyl and maltotriosyl stubs of the beta-limit dextrin are equal and all the maltotriosyl and no maltosyl stubs are removed with the isoamylase. However, the enzyme may hydrolyse some maltosyl stubs of beta-limit dextrin, though only to a limited extent (~7%)[16,17], and maltosyl residues of some oligosaccharides[20]. Therefore, at least 88% of the phosphate groups appear to be located in the B-chain, and the remainder (at most 12%) are bound to the A-chain.

The beta-amylase hydrolysed the iβL-PD partially (first beta-amylolysis), and degraded it further after debranching with pullulanase (second beta-amylolysis) (Table I). This result indicates that beta'- and gamma'-chains are present in iβL-PD, since beta-amylase hydrolyses only a part of the beta'-chain at the first amylolysis and then the debranched gamma'-chain partly at the second amylolysis, as shown in Fig. 4 (the action of the enzyme will be explained later). The sum of the extents of the first and second amylolysis (18%) implies that the molar fraction of the beta'- and the debranched gamma'-chains in five iβL-PD chains is 0.36, assuming that the beta-amylolysis limit of these chains is 50% and that iβL-PD consists of the molecules of equal molecular weight. The phosphate groups of the beta'- and gamma'-chains are originally positioned in the inner section of the B-chains. Accordingly, it implies that 35% of the phosphate groups are located in the position, and the remainder (64%) in the A-chain and the outer section of the B-chain. A similar value was also obtained from the following argument.

PUC (phosphorylated unit-chain derived from potato amylopectin) is a mixture of molecules phosphorylated at C-6 or C-3 of a glucosyl residue. The beta-amylase hydrolyses the substrate having a 6-phosphorylated glucosyl residue until one or no glucosyl residue remains attached thereto by odd or even numbers of glucosyl residues at the non-reducing side[10]. The specificity of action of the enzyme in the vicinity of a

3-phosphorylated glucosyl residue in PUC is unknown, but the enzyme seems to be unable to by-pass the 3-phosphorylated glucosyl residue or expose it at the non-reducing end since the 3-phosphorylated glucosyl residue remained in the beta-limit PUC, and phosphatase treatment of the Smith-degradation products of the beta-limit PUC yielded one mol of glycerol per chain. Thus, the beta-amylase hydrolyses PUC until near or at the phosphorylated residues and exposes a part of the 6-phosphorylated glucosyl residues at the non-reducing end; the beta-limit PUC carried 0.30 mol of 6-phosphorylated glucosyl groups at the non-reducing end per chain (*i.e.*, three chains in 10 had 6-phosphorylated glucosyl groups at the non-reducing end). Similarly, it was found that iβL-PD had 0.21 mol of 6-phosphorylated glucosyl group at the non-reducing end per chain. This indicates that the chains having the phosphate groups at the non-reducing end or the residue close to it, such as the alpha-, beta- and gamma-chains, are present and that the phosphate groups are located in the A-chain and the outer section of the B-chain. The molar fraction of the alpha-, beta-, and gamma-chains in five iβL-PD chains is calculated to be 0.70 from the ratio of 6-phosphorylated glucosyl groups exposed at the non-reducing end of iβL-PD to that of the beta-limit PUC, assuming that the alpha-, beta-, and gamma-chains have the same proportion of exposed 6-phosphorylated glucosyl groups per chain as that of the beta-limit PUC. This value suggests that 70% of the phosphate groups are in the A-chains and the outer section of the B-chains, and generally agrees with the value (64%) from the above-mentioned beta-amylolysis of iβL-PD.

Thus, we conclude that about one third of the phosphate groups are present in the inner sections of the B-chains, and two thirds in the A-chains and the outer sections of the B-chains.

The distribution of the phosphate groups in the amylopectin, namely, no phosphate in the unit chain with a $\overline{\text{d.p.}}$ of less than $\sim$20 (mainly in the B-chain) and more than nine glucosyl residues away from the branch point (Figs. 2 and 3), is of interest for understanding the biosynthesis. This suggests that the enzyme, which is involved in the biosynthesis of amylopectin phosphate, may be specific in its requirements for the size of the unit chain that is phosphorylated or the branch point may interfere sterically with the enzyme, although the mechanism of the biosynthesis is unknown.

EXPERIMENTAL

Preparation of the phosphorylated unit-chain (PUC). — PUC was prepared[10] by the debranching of potato amylopectin with *Pseudomonas* isoamylase. PUC contained $\sim$1 phosphate group (1.08 mol per chain). Of the phosphate groups, 73% were located at C-6 of the glucosyl residues and the remainder at C-3. Beta-limit PUC, which had 0.30 mol of 6-phosphorylated glucosyl group at the non-reducing end per chain, was isolated by ion-exchange chromatography after hydrolysing the PUC with beta-amylase under the conditions used for the preparation of iβL-PD, as described below.

Preparation of the isoamylase-debranched, beta-limit phosphodextrin (iβL-PD). — iβL-PD was prepared by successive hydrolyses of the amylopectin with beta-amylase and the isoamylase, to their limits, as described below. A solution of amylopectin (1 g in 100 mL of 20mM acetate buffer, pH 4.8) was incubated with 2.5×10^4 units of sweet-potato beta-amylase for 23 h at 37°. After heating in a boiling water-bath for 5 min, the pH of the digest was adjusted to 3.5 with acetic acid, and then the digest was incubated with 70 units of the isoamylase for 6 h at 50°. The reaction was terminated by heating, and the resulting iβL-PD was isolated by the procedures used[10] for the preparation of PUC.

Assays. — Reducing sugar, carbohydrate, and 6-phosphorylated glucosyl groups were determined by the methods described elsewhere[21]. Organic phosphate was measured[22] as inorganic phosphate after treatment with hot perchloric acid. The non-reducing end of phosphodextrins was determined by rapid Smith-degradation[23] with minor modifications[10]. 6-Phosphorylated glucosyl groups at the non-reducing end were determined as glycerol after phosphatase treatment of the Smith-degradation products, as described previously[10], since the degradation of 6-phosphorylated glucosyl groups at the non-reducing end yields a mixture of glycerol 3-phosphate and glycerol 2-phosphate because of phosphate migration during the acid hydrolysis[24]. Maltose was assayed[25] with maltose phosphorylase.

Materials. — Potato amylopectin was prepared by the method of Lansky *et al.*[26] in an atmosphere of nitrogen. Crystalline, sweet-potato beta-amylase was prepared by the method described previously[27] and crystallised again from ammonium sulfate solution to provide stability during storage. *Pseudomonas* isoamylase and maltose phosphorylase were gifts from Professor T. Harada (Osaka University) and Dr. K. Yokobayashi (Hayashibara Co. Ltd.), respectively. Crystalline pullulanase (*Aerobacter aerogenes* ATCC 9621) was purchased from Hayashibara Biochemical Research Laboratory.

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