Carbohydrate Research, 61 (1978) 345–357
© Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

ACTION OF *Pseudomonas* ISOAMYLASE ON VARIOUS BRANCHED OLIGO- AND POLY-SACCHARIDES*

KEIJI KAINUMA†, SHOICHI KOBAYASHI†, AND TOKUYA HARADA‡

†*National Food Research Institute, Ministry of Agriculture and Forestry 4-12, Shiohama-1, Koto-ku, Tokyo (Japan)*

‡*The Institute of Scientific and Industrial Research, Osaka University, Yamadakami, Suita-shi, Osaka (Japan)*

(Received July 6th, 1977; accepted for publication in revised form, November 22nd, 1977)

ABSTRACT

Pseudomonas isoamylase (EC 3.2.1.68) hydrolyzes (1 → 6)-α-D-glucosidic linkages of amylopectin, glycogen, and various branched dextrins and oligosaccharides. The detailed structural requirements for the substrate are examined qualitatively and quantitatively in this paper, in comparison with the pullulanase of *Klebsiella aerogenes*. As with pullulanase, *Ps.* isoamylase is unable to cleave D-glucosyl stubs from branched saccharides. *Ps.* isoamylase differs from pullulanase in the following characteristics: (*1*) The favored substrates for *Ps.* isoamylase are higher-molecular-weight polysaccharides. Most of the branched oligosaccharides examined were hydrolyzed at a lower rate, 10% or less of the rate of hydrolysis of amylopectin. (*2*) Maltosyl branches are hydrolyzed off by *Ps.* isoamylase very slowly in comparison with maltotriosyl branches. (*3*) *Ps.* isoamylase requires a minimum of three D-glucose residues in the B- or C-chain.

INTRODUCTION*

Isoamylase is an enzyme that hydrolyzes (1 → 6)-α-D-glucosidic linkages of certain, starch-type, branched oligo- and poly-saccharides. The enzyme was first prepared by Maruo and Kobayashi[1] from yeast. More recently, several microbial, debranching enzymes such as pullulanase[2], *Pseudomonas* isoamylase[3], and *Cytophaga* isoamylase[4] have been prepared, and used for structural analysis of branched oligosaccharides and polysaccharides.

---

*Dedicated to Professor Dexter French on the occasion of his 60th birthday.

*Symbols and abbreviations: G_1,G_2,G_3 ... etc. are D-glucose, maltose, and maltotriose ... etc. B_3,B_4 ... are "branched" oligosaccharides containing a single α-D-(1 → 6)-link. Pan-5, IG_3-5 ... are branched pentasaccharides obtained by the coupling reaction of panose or isomaltotriose. In symbolic formulas, O denotes an α-D-glucopyranose residue, ø denotes a reducing-end D-glucose residue, – is an α-D-(1 → 4) link, and an arrow (↓ or →) between glucose residues is a α-D-(1 → 6) link. The symbol --↓-- is an α-D-(1 → 6) link cleaved by debranching enzymes. *Ps.* isoamylase is *Pseudomonas* isoamylase.

Pseudomonas isoamylase was discovered by one of the authors in 1968. The enzyme was purified to homogeneity and its catalytic properties well characterized[5]. The characteristic feature of *Ps.* isoamylase is that this enzyme completely debranches amylopectin and glycogen[6], which *Klebsiella* pullulanase fails to do. This has enabled *Ps.* isoamylase to contribute to the elucidation of the structure of branched polysaccharides. Mercier and Kainuma[7] reported that the enzyme is stable in 40% dimethyl sulfoxide solution. They suggested that *Ps.* isoamylase would be very useful for determination of the chain-length distribution of the longer, water-insoluble branched polysaccharides (such as amylomaize starch and Type IV storage-disease glycogen) in aqueous dimethyl sulfoxide solution.

Compared with pullulanase[8], the action pattern of *Ps.* isoamylase on branched oligosaccharides has been less well characterized. This paper summarizes our studies of the action of *Ps.* isoamylase on various branched poly- and oligo-saccharides, in comparison with pullulanase.

EXPERIMENTAL

Enzymes. — Porcine-pancreatic alpha amylase was a twice-crystallized preparation (Worthington Biochemical Corp.). Twice-crystallized pullulanase was purchased from Hayashibara Biochemical Research Laboratory, Japan. *Ps.* isoamylase was twice crystallized as by Kato *et al.*[9]. The cycloamylose glycosyl transferase of *Bacillus macerans* was prepared as by Tilden and Hudson[10] and crystallized by Kobayashi[11]. Crude, soybean beta amylase was a gift from Dr. T. Komaki, Nagase Co. (Osaka, Japan). This preparation had no detectable maltase activity, whereas crystalline, sweet-potato beta amylase frequently contains maltase. Furthermore, we did not observe any oligosaccharide except maltose during the reaction. This observation strongly suggested that there was n. alpha amylase activity contaminating the beta amylase preparation.

Polysaccharides and branched oligosaccharides. — Commercial waxy-maize starch was defatted as by T. J. Schoch[12] and used as amylopectin. Oyster glycogen (Fluka A-G.) was purchased from Seikagaku Kogyo Co. Amylopectin (1 g) and oyster glycogen (1 g) were exhaustively treated with 400 U of soybean beta amylase for 24 h to prepare the beta-limit dextrins. After the reaction, the enzyme was inactivated by boiling, and then the digest was dialyzed against distilled water until maltose was completely removed. This procedure was repeated two more times. The last treatment gave no further maltose. The resulting, high-molecular-weight material was a beta-limit dextrin. The degrees of beta amylolysis of amylopectin and glycogen were 58 and 46%, respectively[13]. Pullulan was purchased from Hayashibara Biochemical Laboratory. Singly-branched oligosaccharides (see [c] in Table II) were obtained by the extensive digestion of amylopectin by porcine-pancreatic alpha amylase, and then fractionated by descending paper-chromatography. Pullulan was partially hydrolyzed by pullulanase to obtain multiply branched oligosaccharides in which α-maltotriosyl residues are joined end to end through a (1 → 6)-α-D-glucosidic linkage

(see in Table II [f], and Table III). Isomaltose and isomaltotriose were obtained by acid hydrolysis of dextran. After fractionation, each saccharide was incubated with cyclohexaamylose and *B. macerans* enzyme to generate various branched oligosaccharides[14] terminated at the reducing end by isomaltose or isomaltotriose (Table II, [b], and Table IV). Panose, prepared from hydrol[15], was incubated with cyclohexaamylose and *B. macerans* enzyme to prepare oligosaccharides terminated by panose at the reducing end ([d] in Table II).

The oligosaccharides in the mixtures obtained by amylase digestion or the coupling reaction with *B. macerans* enzyme were fractionated by column chromatography on Biogel P-2[16] or by descending macro paper chromatography with 6:4:4 (v/v) 1-butanol–pyridine–water. The purity of each fraction was confirmed by paper chromatography. The concentrations of poly- and oligo-saccharides were determined by the phenol–sulfuric acid method[17].

Enzyme digestion. — For determination of the initial reaction velocity of the debranching enzymes, the branched oligosaccharides were treated under the following conditions. Substrate [200 µl/mM in the (1 → 6)-α-D-glucosidic bond] was incubated with 10 µl of debranching-enzyme solution [40 mU of *Ps.* isoamylase in 200mM acetate buffer (pH 3.50) or 7 mU of pullulanase in 200mM acetate buffer (pH 5.50)] at 40°. After 10 min, the enzyme was inactivated by boiling and the increase in reducing value was determined by the Somogyi–Nelson method[18]. For amylopectin, glycogen, pullulan, and beta amylase limit dextrins of amylopectin and glycogen, 50 mg of polysaccharide was dissolved in 1 ml of dimethyl sulfoxide by heating at 100°, and then diluted with water to 10 ml to give 0.5% solutions. The relative initial rates calculated from the results are indicated in Tables I–IV where, for isoamylase, 100% was taken as the initial rate of hydrolysis of amylopectin and, for pullulanase, 100% was the initial rate of hydrolysis of pullulan. The reactions were continued for 18 h and then aliquots were spotted onto Toyo filter paper No. 50 to observe the products.

Paper chromatography. — Ascending and descending paper chromatography were conducted on Toyo filter paper No. 50 and Whatman 3 MM with the solvent system 6:4:4 (v/v) 1-butanol–pyridine–water at 60°. In order to survey the action of the enzymes on oligosaccharides, two-dimensional paper chromatography was employed[19]. After irrigation, all of the chromatograms were treated by the glucoamylase dip-method[20] to convert the weakly-reducing oligosaccharides into D-glucose, which was then revealed by the silver nitrate dip-method[21].

RESULTS AND DISCUSSION

Initial rate of reaction of Ps. *isoamylase and pullulanase on branched-chain poly- and oligo-saccharides.* — Relative initial rates were determined by measurement of the increase of reducing value after 10 min of reaction. At this stage of the reaction, less than 10% of the α-D-(1 → 6) bonds of substrates were hydrolyzed, and the reaction rate was linear. The results are shown in Tables I–IV.

TABLE I

RELATIVE REACTION-RATES[a] OF *Pseudomonas* ISOAMYLASE AND PULLULANASE ON VARIOUS BRANCHED POLYSACCHARIDES

Substrates	Ps. *isoamylase*	*Pullulanase*
Amylopectin	100	15
Beta-limit dextrin of amylopectin	80.3	56.9
Oyster glycogen	124	1
Beta-limit dextrin of oyster glycogen	214	10.2
Pullulan	<1	100

[a]Relative reaction rates of the enzymes were determined by the increase in reducing value, as described in the Experimental section.

TABLE II

RELATIVE REACTION-RATES[a] OF *Pseudomonas* ISOAMYLASE AND PULLULANASE ON VARIOUS SINGLY BRANCHED OLIGOSACCHARIDES

Oligosaccharides			*Relative reaction rates*	
	Name	*Structure*	Ps. *isoamylase*	*Pullulanase*
1	Panose	0 ↓ 0-ø	0	0
2	Isopanose[b]	0-0 ↓ ø	0	0
3	6[1]-*O*-α-D-Glucosyl-maltose[b]	0 ↓ 0-0	0	0
4	6[3]-*O*-α-D-Glucosyl-maltotriose[c]	0 ↓ 0-0-ø	0	0
5	6[2]-*O*-α-Maltosyl-maltose[d]	0-0 ↓ 0-ø	0	2
6	6[2]-*O*-α-D-Glucosyl-maltotriose[d]	0 ↓ 0-0-ø	0	0
7	6[1]-*O*-α-Maltosyl-maltose[b]	0-0 ↓ 0-ø	0	0
8	6[3]-*O*-α-D-Glucosyl-maltotetraose[c]	0 ↓ 0-0-0-ø	0	0
9	6[3]-*O*-α-Maltosyl-maltotriose[c]	0-0 ↓ 0-0-ø	2.8	22
10[e]	6[2]-*O*-α-Maltosyl-maltotriose[d] or 6[2]-*O*-α-maltotriosyl-maltose	0-0 or 0-0-0 ↓ or ↓ 0-0-ø 0-ø	0	8.6

TABLE II *(continued)*

Oligosaccharides			*Relative reaction rates*	
	Name	*Structure*	Ps. *isoamylase*	*Pullulanase*
11	6^1-*O*-α-Maltotriosyl-maltose[b]	0-0-0 ↓ 0-ø	0	0
12	6^3-*O*-α-Maltosyl-maltotetraose[c]	0-0 ↓ 0-0-0-ø	6.9	43
13	6^3-*O*-α-Maltotriosyl-maltotriose[f]	0-0-0 ↓ 0-0-ø	9.7	162
14[e]	6^2-*O*-α-Maltotriosyl-maltotriose[d] and 6^2-*O*-α-maltotetraosyl-maltose[d]	0-0-0 and 0-0-0-0 ↓ and ↓ 0-0-ø 0-ø	2.7	56
15	6^3-*O*-α-Maltotriosyl-maltotetraose[c]	0-0-0 ↓ 0-0-0-ø	33	146
16	6^3-*O*-α-Maltosyl-maltopentaose[c]	0-0 ↓ 0-0-0-0-ø	8.3	98
17	6^2-*O*-α-Maltopentaosyl-maltose[d]	0-0-0-0-0 ↓ 0-ø	0	26
18	6^2-*O*-α-Maltotetraosyl-maltotriose[d]	0-0-0-0 ↓ 0-0-ø	6.8	26
19[e]	6^3-*O*-α-Maltotriosyl-maltopentaose[c] and 6^3-*O*-α-maltotetraosyl-maltotetraose[c]	0-0-0 and 0-0-0-0 ↓ and ↓ 0-0-0-0-ø 0-0-0-ø	18	86
	Pullulan		<1	100
	Amylopectin		100	15

[a]See footnote [a], Table I. [b]Obtained by the coupling reaction of *B. macerans* enzyme on isomaltose. [c]Obtained from amylopectin by digestion with porcine-pancreatic alpha amylase. [d]Obtained by the coupling reaction of *B. macerans* enzyme on panose. [e]The oligosaccharides **10**, **14**, and **19** were mixtures of isomers that we were unable to separate. [f]Obtained by partial hydrolysis of pullulan by pullulanase alone, or by a combination of pullulanase and beta amylase.

As shown in Table I, *Ps.* isoamylase hydrolyzed oyster glycogen and its beta amylase limit dextrin at relative rates of 124 and 214, respectively. In comparison, pullulan was hydrolyzed extremely slowly (< 1). As already stated[22,23], amylopectin and glycogen were very slowly hydrolyzed by pullulanase, at relative rates of 15 and 1. The beta amylase limit dextrins of these polysaccharides were attacked much more rapidly, with relative rates of 80 and 214.

Because of the lack of a common substrate hydrolyzed by the two enzymes at almost the same rate, we used different reference polysaccharides: pullulan for pullulanase, and amylopectin for *Ps.* isoamylase.

The results of the determinations of relative initial-rate for various branched oligosaccharides are shown in Table II. There was no reaction for oligosaccharides **1** to **8** (Table II) by either enzyme, except for a very slow hydrolysis of tetrasaccharide **5** by pullulanase. Although **7** and **11** have been reported by Abdullah and French to be hydrolyzed[24], we did not observe any reaction under the conditions employed in this work. Rates of hydrolysis for **7** and **11** are probably less than 1% when compared with pullulan, and we have neglected rates less than 1% in Tables I–IV.

As shown in Table II, **13** and **15** were better substrates for pullulanase, which hydrolyzed them at relative rates of 162 and 146, respectively. Compounds **15** and **19** were hydrolyzed by isoamylase at higher rates than the other oligosaccharides. The other branched oligosaccharides were hydrolyzed at rates less than 10% of that of amylopectin.

Compound **12** was degraded by pullulanase at a rate different from the values reported by Abdullah[8] and Walker[25]. This difference seems to be attributable to the purity of the branched oligosaccharide having d.p. 6. For our preparation of **12**, the B_6 fraction of porcine-pancreatic, alpha amylase limit-dextrin (a mixture of **12** and **13**) was treated with *Ps.* isoamylase to eliminate as much **13** as possible and to obtain a relatively resistant fraction. By this treatment, the rate was decreased by

TABLE III

RELATIVE REACTION-RATES[a] OF *Pseudomonas* ISOAMYLASE AND PULLULANASE ON MULTIPLY BRANCHED OLIGOSACCHARIDES OBTAINED BY ENZYMIC HYDROLYSIS OF PULLULAN

Oligosaccharide		*Relative reaction rates*	
Number	*Structure*	Ps. *Isoamylase*	*Pullulanase*
13	0-0-0[b] ↓ 0-0-ø	9.7	162
20	0[b] ↓ 0-0-0 ↓ 0-0-ø	3	40
21	0-0-0[b] ↓ 0-0-0 ↓ 0-0-ø	4.2	116
22	0-0-0[b] ↓ $(0\text{-}0\text{-}0)_2$ ↓ 0-0-ø	4.1	110
Amylopectin		100	15
Pullulan		<1	100

[a]Relative reaction-rates were determined as described in Table I. [b]Obtained by partial hydrolysis of pullulan by pullulanase alone, or by a combination of pullulanase and beta amylase.

about one third, and we observed mainly $G_2 + G_4$ and only a very small proportion of G_3 in the digest. Pure **13** was obtained by partial hydrolysis of pullulan by pullulanase.

Generally, the oligosaccharides were hydrolyzed very slowly by isoamylase when compared with the hydrolysis of amylopectin or glycogen.

Branched oligosaccharides derived from pullulan (**13**, **20**, **21**, and **22**) were obtained by partial hydrolysis of pullulan by pullulanase alone or by the combination of pullulanase and beta amylase (Table III). Compound **20** was hydrolyzed by pullulanase at 40% of the rate of pullulan hydrolysis. On the other hand, compounds **13**, **21**, and **22** were hydrolyzed faster than pullulan. Whereas **13** was hydrolyzed at a relative rate of 162, the rates were decreased to 116 and 110 for the larger substrates. *Ps.* isoamylase hydrolyzed **20**, **21**, and **22** at only 3–4% the rate of amylopectin, although **13** was degraded twice as fast as **20**, **21**, and **22**.

Product specificity of Ps. *isoamylase and pullulanase.* — The product specificity of each enzyme was qualitatively examined by paper chromatography. As is clearly shown in Fig. 1, G_2 and G_3 were formed from beta-limit dextrins of amylopectin and glycogen by the action of both debranching enzymes. We did not observe any significant differences in the product specificities of branched oligo- and poly-saccharides between *Ps.* isoamylase and pullulanase in terms of the length of the branch, although the *Ps.* isoamylase cleaved maltosyl branches more slowly than maltotriosyl branches (see Table II). There was no reaction by either enzyme on oligosaccharides having D-glucosyl stubs.

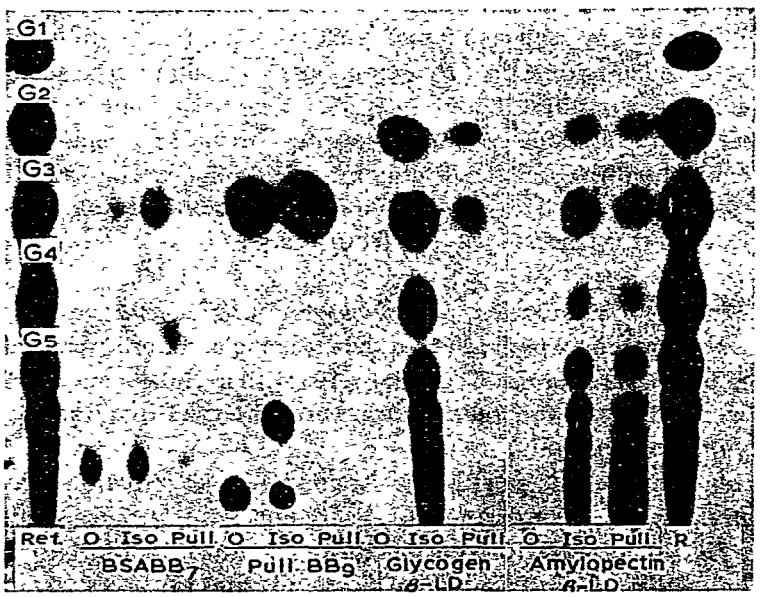


Fig. 1. Paper chromatogram showing action of debranching enzymes on various substrates (I). Ref., reference oligosaccharides; O, substrate without enzyme; Iso., treated by *Ps.* isoamylase; Pull., treated by pullulanase. $BSABB_7$ is oligosaccharide **20**, Pull. BB_9 is **21**, β-LD is beta amylase limit-dextrin.

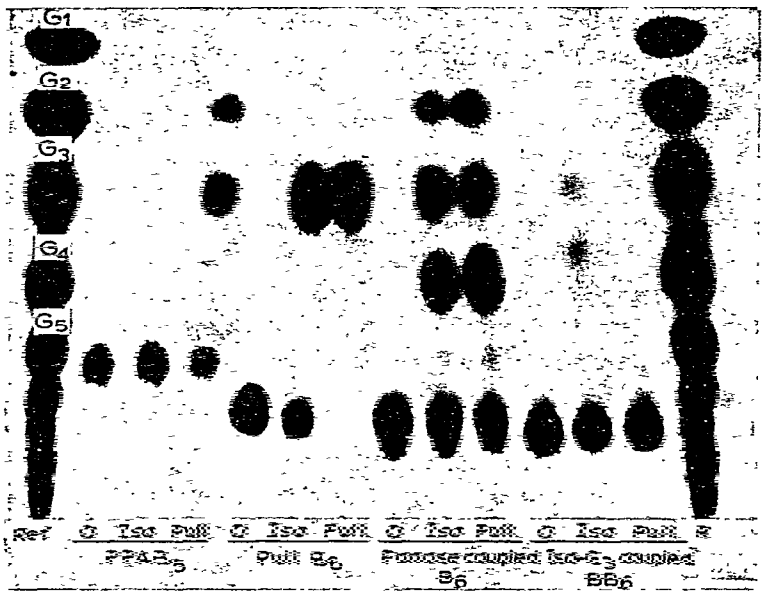


Fig. 2. Paper chromatogram showing action of debranching enzymes on various substrates (II). Ref., O, Iso., and Pull. are as in Fig. 1. $PPAB_5$ is **9**, Pull. B_6 is **13**, Panose-coupled B_6 is **14**, and IG_3-coupled BB_6 is **25**.

Ps. isoamylase seemed to differ from *Cytophaga* isoamylase in its ability to split maltosyl branches from the beta-limit dextrins of glycogen and amylopectin. It was reported that *Cytophaga* isoamylase was unable to cleave a maltosyl stub from beta-limit dextrin of glycogen and amylopectin[4]. *Ps.* isoamylase and pullulanase acted on all of the substrates examined in Figs. 1 and 2. It was clear that maltose and maltotriose were reaction products from several substrates, including beta-limit dextrins. *Ps.* isoamylase reacted with **9** and the doubly branched oligosaccharide **20**, which was obtained both from a pullulan hydrolyzate and from a bacterial, saccharifying alpha amylase limit-dextrin. It formed $G_2 + G_3$ from **9**, and $G_3 + B_4$ from **20**, respectively.

Action of the debranching enzymes on panose-coupled oligosaccharides. — Branched oligosaccharides terminated by panose (**1**) at the reducing end were prepared by the coupling reaction of *B. macerans* enzyme (EC 2.4.1.19) with cyclohexaamylose. The action patterns of *Ps.* isoamylase and pullulanase were observed qualitatively by two-dimensional paper chromatography.

Fig. 3 shows the reaction products with *Ps.* isoamylase. No reaction was observed for **10**. Compound **14** was degraded to $G_3 + G_3$ and an extremely small proportion of $G_2 + G_4$; $G_3 + G_4$ were formed from **18**. As shown in Fig. 4, G_2 and G_3 were formed by pullulanase from Pan-5 (**10**), $G_3 + G_3$ and $G_4 + G_2$ from Pan-6 (**14**), and $G_2 + G_5$ and $G_4 + G_3$ from **17** and **18**. Oligosaccharides terminated by the panose structure in the reducing end were hydrolyzed at lower rates by *Ps.* isoamylase than by pullulanase. This lower reaction rate could be attributed to the

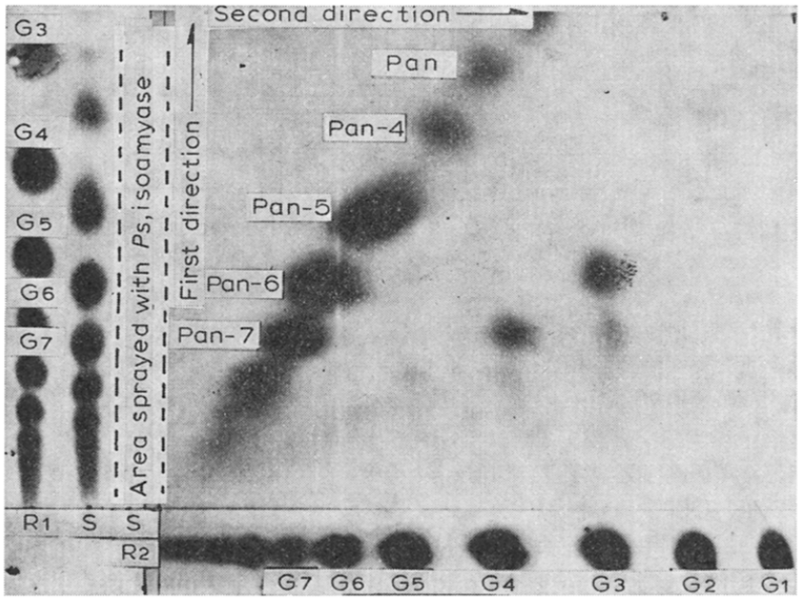


Fig. 3. Two-dimensional paper chromatogram showing action of *Ps.* isoamylase on panose-coupled oligosaccharides. R_1,R_2 are reference series for the first and second direction of the chromatograms; *S* is the point of application of the sample. After irrigation in the first direction, the left side of the chromatogram containing R_1 and one of the S channels was cut off for reference. The remaining *S* channel was sprayed with the enzyme solution. After allowing enzyme action on the paper, the chromatogram was dried, reference R_2 was applied, and the chromatogram was developed in the vertical direction. Pan-4, Pan-5 ... are tetrasaccharide, pentasaccharide ... terminated by panose at the reducing end.

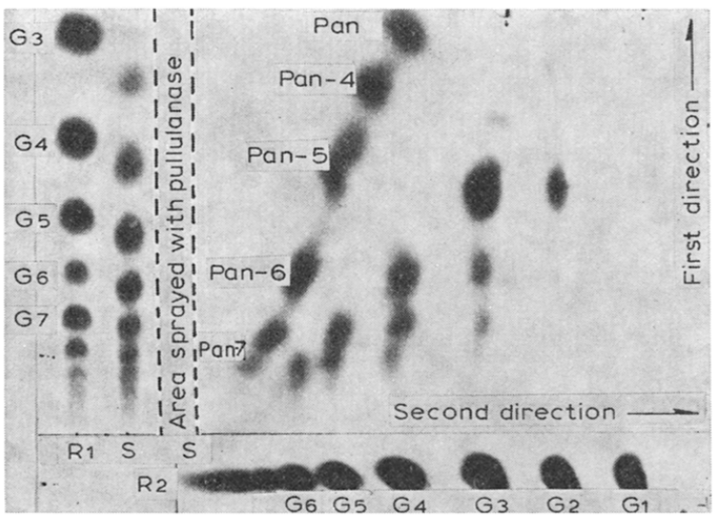


Fig. 4. Two-dimensional paper chromatogram showing pullulanase action on panose-coupled oligosaccharides. Symbols and methods are as in Fig. 3.

number of D-glucose residues attached to the C-chain of oligosaccharides. This will be discussed later.

Action of the debranching enzymes on the isomaltotriose-coupled oligosaccharides. — As shown in Table II, no reaction was observed from the action of pullulanase and *Ps.* isoamylase on such oligosaccharides terminated by isomaltose at the reducing end as **2**, **3**, **7**, and **11**, under the conditions employed in this study, either by test-tube reactions or by two-dimensional paper chromatography. Isomaltose, isomaltotriose, and isomaltotetraose were totally resistant to hydrolysis by both debranching enzymes.

Some of the isomaltotriose-coupled oligosaccharides were hydrolyzed by pullulanase, as shown in Fig. 5. G_3 + isomaltose and G_2 + B_3 were obtained from the IG_3-5 fraction (**23** and **24**). The relative rate of the reaction was 2 as compared with 100 for pullulan by a separate test-tube reaction. G_3 + B_3 and G_2 + B_4 were obtained from the IG_3-6 (**25** and other saccharides) fraction at a rate of 13. The possible structures of these oligosaccharides are shown in Table IV. One of the α-D-(1 → 6) bonds in the isomaltotriose unit has been written another way[13,26] to show the structural analogy with panose-coupled oligosaccharides **10** and **14**. The evidence suggests that pullulanase accepts α-D-(1 → 6)-bonds in place of α-D-(1 → 4)-bonds

TABLE IV

ACTION OF *Pseudomonas* ISOAMYLASE AND PULLULANASE ON ISOMALTOTRIOSE-COUPLED OLIGOSACCHARIDES

Isomaltotriose-coupled oligosaccharides				*Panose-coupled oligosaccharides*			
Number	*Structure*			*Number*	*Structure*		
23	0-0-0 ↓ 0 ↓ ø	≡ 0-0-0[a] --↓-- 0→ø		**10**	0-0-0 --↓-- 0-ø		
		0[b]	2[c]		0[b]	8.6[c]	
24	0-0 ↓ 0-0 ↓ ø	≡ 0-0[a] --↓-- 0-0→ø		**10**	0-0 --↓-- 0-0-ø		
		0[b]	2[c]		0[b]	8.6[c]	
25	0-0-0 ↓ 0-0 ↓ ø	≡ 0-0-0[a] --↓-- 0-0→ø		**14**	0-0-0 --↓-- 0-0-ø		
		+0[b]	13[c]		2.7[b]	56[c]	

[a]Rewritten form of isomaltotriose-coupled oligosaccharides. [b]Relative rates with *Ps.* isoamylase. [c]Relative rates with pullulanase.

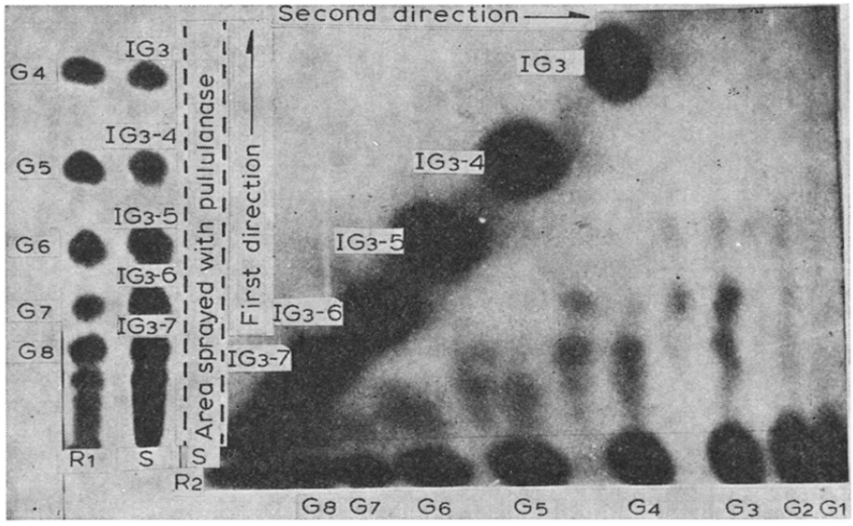
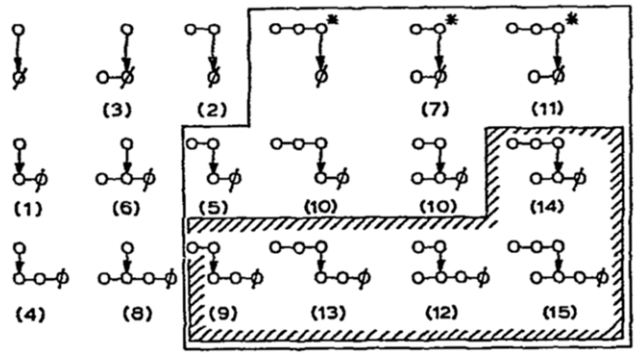


Fig. 5. Two-dimensional paper chromatogram showing pullulanase-coupled oligosaccharides. Symbols and methods are as in Fig. 3. IG_3. is isomaltotriose, IG_3-4, IG_3-5 ... are abbreviations for the branched tetra-, penta-, and higher saccharides terminated by isomaltotriose at the reducing end.



= Susceptible to pullulanase ;

= Susceptible to pullulanase and Ps isoamylase ;

* Susceptibility has been reported qualitatively elsewhere[24,27]

Fig. 6. Susceptibility of branched oligosaccharides to *Pseudomonas* isoamylase and pullulanase in relation to their structures.

at the right-hand side of the cleavage point. In comparison with panose-coupled oligosaccharides (Table IV), the oligosaccharides possessing isomaltotriose structures are always cleaved at lower rates.

Relation between branched structures and susceptibility of oligosaccharides to Ps. *isoamylase and pullulanase.* — The oligosaccharides terminated by isomaltose, panose, and 6^3-α-D-glucosyl-maltotriose at the reducing end were collected from

Table II and are arranged in Fig. 6 to display a generalization for the susceptibility of hydrolysis and the structure of the oligosaccharides. The oligosaccharides from **1** to **15** (Table II), plus isomaltose and 6-*O*-α-maltotriosyl-D-glucose, are listed in Fig. 6. Although we could not observe quantitatively the reaction of 6-*O*-α-maltotriosyl-D-glucose and the tetrasaccharides **7** and **11** by pullulanase in this study, we assumed that these oligosaccharides were hydrolyzed at a rate of less than 1% as compared with that of pullulan. We included these oligosaccharides as susceptible to pullulanase inasmuch as Kainuma and French[27] reported the reaction of pullulanase on reducing-end, ^{14}C-labeled 6-*O*-α-maltotriosyl-D-glucose, radioactive D-glucose being a product, and Abdullah and French[24] reported the cleavage of **7** and **11**.

From our results, the smallest substrates for pullulanase are the branched tetrasaccharides **7**, **5**, and 6-*O*-α-maltotriosyl-D-glucose, as stated by former workers[8,24]. On the other hand, the smallest substrate for *Ps.* isoamylase is the pentasaccharide **9**. *Ps.* isoamylase requires maltotriose or a longer structure in the C-chain of oligosaccharides. A maltosyl branch in the A-chain was always cleaved at a lower rate than a maltotriosyl branch. The minimum substrate-requirement of this enzyme seemed to be a maltose (or preferably larger) A-chain and at least a maltotriosyl group in the B or C-chain at the point of cleavage. As may be seen in Table II and Fig. 6, the addition of one D-glucosyl group to **9** at the non-reducing end of the A-chain increased by 3.5 times the rate for **13**. The addition of one D-glucosyl group to **9** at the non-reducing end of the C-chain increased the rate for **12** by 2.5 times. When one D-glucosyl group was added to **9** at the non-reducing ends of the A- and the C-chains, the rate for **15** was increased by 12 times as compared with the pentasaccharide **9**.

ACKNOWLEDGMENTS

The authors thank Dr. Shigeo Suzuki for his continuous encouragement of this work. We also thank Professor Dexter French, Iowa State University, for his valuable discussion concerning this work, and Dr. John F. Robyt, Iowa State University, for his help, especially in improving the writing of this manuscript.

REFERENCES

1 B. MARUO AND T. KOBAYASHI, *Nippon Nogei Kagaku Kaishi*, 23 (1949) 115–119.
2 H. BENDER AND K. WALLENFELS, *Biochem. Z.*, 334 (1961) 79–95.
3 T. HARADA, K. YOKOBAYASHI, AND A. MISAKI, *Appl. Microbiol.*, 16 (1968) 1439–1444.
4 Z. GUNJA-SMITH, J. J. MARSHALL, E. E. SMITH, AND W. J. WHELAN, *FEBS Lett.*, 12 (1970) 96–100.
5 K. YOKOBAYASHI, A. MISAKI, AND T. HARADA, *Biochim. Biophys. Acta*, 212 (1970) 458–469.
6 T. HARADA, A. MISAKI, H. AKAI, K. YOKOBAYASHI, AND K. SUGIMOTO, *Biochim. Biophys. Acta*, 268 (1972) 497–505.
7 C. MERCIER AND K. KAINUMA, *Stärke*, 27 (1975) 289–292.
8 M. ABDULLAH, B. J. CATLEY, E. Y. C. LEE, J. F. ROBYT, K. WALLENFELS, AND W. J. WHELAN, *Cereal Chem.*, 43 (1966) 111–118.
9 K. KATO, Y. KONISHI, A. AMEMURA, AND T. HARADA, *Agric. Biol. Chem.*, in press.
10 F. F. TILDEN AND C. S. HUDSON, *J. Bacteriol.*, 43 (1942) 527–544.

11 S. KOBAYASHI, K. KAINUMA, AND S. SUZUKI, *Carbohydr. Res.*, 61 (1978) 229–238.
12 T. J. SCHOCH, *Methods Carbohydr. Chem.*, 4 (1964) 56–61.
13 K. KAINUMA, K. WAKO, S. KOBAYASHI, A. NOGAMI, AND S. SUZUKI, *Biochim. Biophys. Acta*, 410 (1975) 333–346.
14 D. FRENCH, M. L. LEVINE, E. NORBERG, P. NORDIN, J. H. PAZUR, AND G. M. WILD, *J. Am. Chem. Soc.*, 76 (1954) 2387–2390.
15 K. KAINUMA, K. SUGAWARA-HATA, AND S. SUZUKI, *Stärke*, 26 (1974) 274–277.
16 K. KAINUMA, A. NOGAMI, AND C. MERCIER, *J. Chromatogr.*, 121 (1976) 361–369.
17 M. DUBOIS, J. K. GILLES, R. A. HAMILTON, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356.
18 N. NELSON, *J. Biol. Chem.*, 153 (1944) 375–380.
19 D. FRENCH, A. O. PULLEY, M. ABDULLAH, AND J. C. LINDEN, *J. Chromatogr.*, 24 (1966) 271–276.
20 K. KAINUMA AND D. FRENCH, *FEBS Lett.*, 5 (1969) 257–261.
21 J. F. ROBYT AND D. FRENCH, *Arch. Biochem. Biophys.*, 100 (1963) 451–467.
22 K. YOKOBAYASHI, H. AKAI, T. SUGIMOTO, M. HIRAO, K. SUGIMOTO, AND T. HARADA, *Biochim. Biophys. Acta*, 293 (1973) 197–202.
23 E. Y. C. LEE AND W. J. WHELAN, *Enzymes*, 3rd edn., (1971) 200.
24 M. ABDULLAH AND D. FRENCH, *Arch. Biochem. Biophys.*, 137 (1970) 483–493.
25 G. J. WALKER, *Biochem. J.*, 108 (1968) 33–40.
26 N. Y. GIRI AND D. FRENCH, *Arch. Biochem. Biophys.*, 145 (1971) 505–510.
27 K. KAINUMA AND D. FRENCH, *Abstr. Pap. Jpn. Agric. Chem. Soc. Meet.*, (1971) 214.