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Biosynthesis of curdlan from culture media containing ^{13}C-labeled glucose as the carbon source

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ABSTRACT

^{13}C-Labeled curdlans were biosynthesized by *Agrobacterium* sp. (ATCC 31749) from culture media containing D-(1-^{13}C)glucose, D-(6-^{13}C)glucose, or D-(2-^{13}C)glucose as the carbon source, and their structures were analyzed by ^{13}C NMR spectroscopy. The labeling was mainly found in the original position, that is, C-1, C-6, or C-2, indicating direct polymerization of introduced glucose. In addition, C-3 in curdlan obtained from D-(1-^{13}C)glucose, C-1 in curdlan obtained from D-(6-^{13}C)glucose, and C-1 and C-3 in curdlan obtained from D-(2-^{13})glucose were labeled. From analysis of this labeling, the biosynthesis of curdlan was interpreted as involving five routes: (*1*) direct synthesis from glucose; (*2*) rearrangement (1-^{13}C → 3-^{13}C); and (*3*) isomerization (6-^{13}C → 1-^{13}C) of cleaved trioses by the Embden–Meyerhof pathway, followed by neogenesis of glucose and formation of curdlan; (*4*) from fructose 6-phosphate formed in the pentose cycle (2-^{13}C → 1-^{13}C, 3-^{13}C); and (*5*) neogenesis of glucose from fragments produced in various pathways of glycolysis. The ^{13}C-labeling at C-6 and C-2 in the starting glucoses is well preserved in the C-6 carbon and the C-1 to C-3 carbons, respectively, in the curdlan produced.

INTRODUCTION

Curdlan [(1 → 3)-β-D-glucan] is an extracellular polysaccharide produced by *Alcaligenes faecalis* var. *Myxogenes*[1]. This polysaccharide is commonly found in

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yeasts, fungi, and higher plants as a constituent of cell walls or as a reserve polysaccharide.

Curdlan is also used as a food additive for gelling. Such naturally occurring branched (1 → 3)-β-D-glucans as lentinan and schizophyllan have been shown to have antitumor activity[2]. These branched polysaccharides have been produced from curdlan and also showed high antitumor activity[3,4]. Furthermore, it was reported recently that sulfates of curdlan and its branched derivatives exhibit high anti-HIV (AIDS virus) activity in vitro[5–8].

The biochemical pathway for formation of curdlan has not been investigated. For cellulose and starch, several investigations on their biochemical formation using ^{14}C- and ^{13}C-labeled glucoses or other low-molecular-weight compounds as carbon sources have been reported[9–14]. Herein we have investigated the biosynthesis of curdlan using ^{13}C-labeled glucoses, in order to throw light on the mechanism of its biosynthesis.

EXPERIMENTAL

^{13}C-Labeled glucose.—As the labeled carbon source, D-(1-^{13}C)glucose, D-(6-^{13}C)glucose, and D-(2-^{13}C)glucose (Isotec Inc., OH, USA) were used. The isotopic purity of the labeled glucoses was 99.0, 98.7, and 99.3%, respectively, as determined by mass spectrometry of their diacetone derivatives.

Activation of bacterium.—Preserved *Agrobacterium* sp. (ATCC 31749) culture was inoculated into PY medium (distilled water, 100 mL; peptone, 1.0 g; yeast extract, 0.5 g; NaCl, 0.5 g; pH 7.2)[15] and incubated for 3 days at 28°C. The activated bacterium was then inoculated on agar medium[16] (distilled water, 100 mL, D-glucose, 2.0 g; $(NH_4)_2HPO_4$, 0.23 g; KH_2PO_4, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; $FeSO_4 \cdot 7H_2O$, 5 mg; $MnSO_4 \cdot H_2O$, 2 mg; $ZnCl_2$, 1 mg; $CoCl_2$, 1 mg; $CaCO_3$, 0.3 g; uracil, 0.01 g; agar, 2.0 g) and incubated for 3 days at 28°C.

Starter culture.—A selected colony having a high productivity of polysaccharide was inoculated into a medium[16] containing distilled water (100 mL), D-glucose (8.0 g), $(NH_4)_2HPO_4$ (0.23 g), KH_2PO_4 (0.1 g), $MgSO_4 \cdot 7H_2O$ (0.05 g), $FeSO_4 \cdot 7H_2O$ (5 mg), $MnSO_4 \cdot H_2O$ (2 mg), $ZnCl_2$ (1 mg), $CoCl_2$ (1 mg), $CaCO_3$ (0.3 g), and uracil (0.01 g)[16] and incubated with shaking at 120 strokes/min for 2 days at 30°C.

Production of ^{13}C-labeled curdlan.—The incubated medium (5 mL) was added to fresh medium (95 mL) with the same components as the starter culture, except for D-glucose. D-Glucose used for production of labeled curdlan contained 10 wt% of D-(1-^{13}C)glucose, 5 wt% of D-(6-^{13}C)glucose, or 5 wt% of D-(2-^{13}C)glucose. The culture was incubated for 7 days under conditions similar to those used for the starter culture. The product was precipitated from the medium by centrifugation (8000 rpm for 15 min at 4°C), washed with 2 M aq HCl to remove $CaCO_3$, and rinsed with distilled water until acid-free. The sediment was then dissolved in 0.5 M aq NaOH and the bacterium was removed from the solution by centrifugation (13000 rpm for 30 min at 4°C). The supernatant was neutralized with 1 M aq HCl

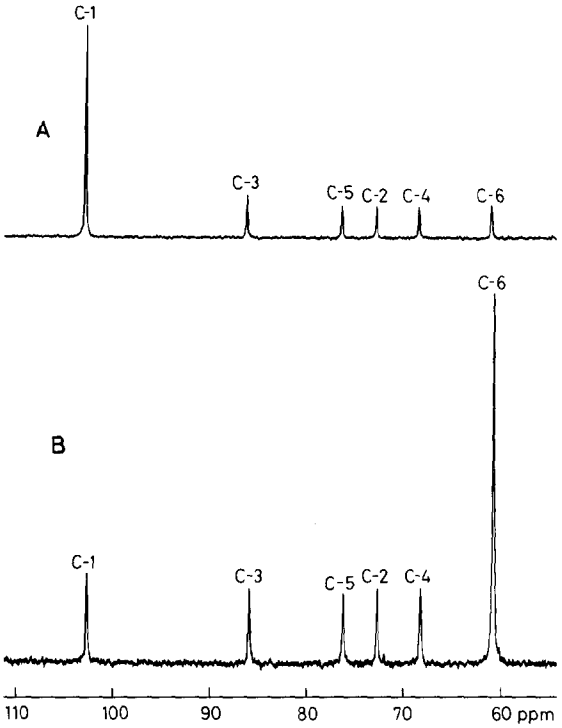

Fig. 1. ^{13}C NMR spectra of curdlans obtained from culture media containing: (A) 10 wt% of D-(1-^{13}C)glucose, measured with nongated decoupling; (B) 5 wt% of D-(6-^{13}C)glucose, measured with gated decoupling.

and curdlan was precipitated by centrifugation (10000 rpm for 20 min at 4°C). Curdlan was washed thoroughly with distilled water and dried under vacuum; yields 0.1–0.2 g of curdlan per 1.0 g of glucose.

^{13}C NMR spectroscopy.—The ^{13}C NMR spectra of curdlan were recorded for solutions in $(CD_3)_2SO$ at 60°C with a Jeol EX 270 spectrometer at 67.80 MHz. A repetition time of 2.0 s (30000 scans) was used for measurements with nongated decoupling, and a repetition time of 10.0 s (17000 scans) was used for measurements with gated decoupling. Chemical shifts were referenced to internal $(CD_3)_2SO$ (39.50 ppm).

RESULTS AND DISCUSSION

Fig. 1 shows the ^{13}C NMR spectra of curdlans obtained from cultures containing D-(1-^{13}C)glucose or D-(6-^{13}C)glucose as the carbon source (the spectrum of curdlan obtained from D-(2-^{13}C)glucose is not shown). Table I indicates the ^{13}C enrichment ratio (ER, the ratio of introduced ^{13}C carbon atom intensity, that is, observed ^{13}C carbon intensity minus natural-abundance ^{13}C carbon intensity, to

TABLE I

^{13}C Enrichment ratio (ER), and labeled ratio (LR) for each carbon in curdlans obtained from labeled glucoses [a]

Chemical shift	C-1 (102.8)	C-2 (72.6)	C-3 (86.0)	C-4 (68.2)	C-5 (76.2)	C-6 (60.7)	Total
Curdlan from D-(1-^{13}C)glucose							
ER	5.59	0.11	0.30	0	0	0.02	6.02
LR	57.0%	1.1%	3.3%	0	0	0	61.4%
	(93.2%)	(1.8%)	(5.0%)	(0)	(0)	(0)	(100%)
Curdlan from D-(6-^{13}C)glucose							
Gated decoupling (without NOE enhancement)							
ER	0.41	0	0.08	0	0	3.97	4.46
LR	8.4%	0	1.6%	0	0	81.2%	91.2
	(9.3%)	(0)	(1.4%)	(0)	(0)	(89.3%)	(100%)
Nongated decoupling (with NOE enhancement)							
ER	0.35	0.09	0	0.07	0.04	3.96	4.51
LR	7.2%	1.8%	0	1.4%	0	81.0%	92.2%
	(7.8%)	(1.9%)	(0)	(1.5%)	(0)	(89.8%)	(100%)
Curdlan from D-(2-^{13}C)glucose							
ER	0.95	3.02	0.53	0	0	0	4.53
LR	19.3%	61.3%	10.8%	0	0	0	91.5%
	(21.1%)	(67.0%)	(11.8%)	(0)	(0)	(0)	(100%)

[a] Purity of D-(1-^{13}C)glucose, D-(6-^{13}C)glucose, and D-(2-^{13}C)glucose was 99.0, 98.7, and 99.3%, respectively. The culture media contained 10, 5, and 5% of D-(1-^{13}C)glucose, D-(6-^{13}C)glucose, and D-(2-^{13}C)glucose, respectively. Values in parentheses under LR values indicate percentages of introduced ^{13}C atoms for each carbon atom in the total ^{13}C introduced.

natural-abundance ^{13}C carbon atom intensity), calculated from peak areas in Fig. 1. Table I also indicates the labeling ratio (LR) of each carbon. LR is the ratio of ^{13}C carbon atoms introduced into the polymer to the ^{13}C carbon atom intensity of the C-1, C-6, or C-2 carbon used for the ^{13}C carbon source. Therefore, if the starting glucose polymerizes directly to curdlan, the LR of C-1, C-6, or C-2 would be 100%. Total LR indicates the total proportion of ^{13}C introduced into the polymer. For calculating LR, the isotopic purities of D-(1-^{13}C)glucose, D-(6-^{13}C)glucose and D-(2-^{13}C)glucose were assumed to be 99.0, 98.7, and 99.3%, respectively, as stated in the Experimental.

For C-6-labeled curdlan, ^{13}C NMR measurements were carried out both with nongated decoupling and gated decoupling. The peak areas determined with nongated decoupling were corrected by the peak areas of unlabeled curdlan determined under the same conditions. It is evident from Table I that both measurements agree well. This indicates that measurements with nongated decoupling, which are time saving, give the same results as those with gated decoupling when the correction is carried out.

Table I shows that the ^{13}C carbon atoms introduced are mainly found in the original position, 93% of the introduced ^{13}C carbon at C-1, 89% at C-6, and 67%

at C-2, as observed in the biosynthesis of cellulose[9–14]. It should be noted that in curdlans obtained from D-(6-^{13}C)glucose and D-(2-^{13}C)glucose the total LR is very high (91–92%), while it is 61% for curdlan obtained from D-(1-^{13}C)glucose.

In curdlan obtained from D-(1-^{13}C)glucose, a small amount of labeling at C-3 carbon was observed, as observed with cellulose from *Acetobacter xylinum*[10]. This process can be explained by rearrangement of (1-^{13}C)dihydroxyacetone 1-phosphate to (3-^{13}C)dihydroxyacetone 1-phosphate by the Embden–Meyerhof pathway, followed by recombination of trioses to glucose. It is very probable that an enzyme catalyzes the rearrangement, although such an enzymic reaction is not well known.

The cause of dilution of total labeling to 61% in curdlan obtained from D-(1-^{13}C)glucose may be ascribed to isomerization of the unlabeled C-6 or C-2 carbon to C-1 carbon via the Embden–Meyerhof pathway or the pentose cycle[17], and Entner–Doudoroff pathway, which result in the loss of C-1 labeling, as discussed later. The importance of the Entner–Doudoroff pathway in *Agrobacterium* has been discussed[18,19].

In curdlan obtained from D-(6-^{13}C)glucose, considerable labeling at C-1 was observed, as is the case in cellulose produced by *Acetobacter xylinum*[10,13] and cellulose obtained from the cotton boll[11,12]. This phenomenon has been explained by reversible isomerization of D-(3-^{13}C)glyceraldehyde 3-phosphate to (1-^{13}C)dihydroxyacetone 1-phosphate[12]. However, it should be noted that ^{13}C labeling at C-6 was not observed in curdlan obtained from D-(1-^{13}C)glucose. The reason may be that the concentration of D-(3-^{13}C)glyceraldehyde 1-phosphate formed by isomerization of (1-^{13}C)dihydroxyacetone 1-phosphate is very low [presumably 1–2%, when estimated from C-6 → C-1 labeling transfer (8%) and polymer yield (10–20%)], while unlabeled D-glyceraldehyde 3-phosphate is formed in the pentose cycle, the Entner–Doudoroff pathway, and the Embden–Meyerhof pathway. Thus, reconstructed glucose will have low labeling at C-6, unless labeled D-[3-^{13}C]glyceraldehyde 3-phosphate reacts with dihydroxyacetone 1-phosphate to form labeled glucose immediately after its formation.

A high LR value (81%) at C-6 clearly indicates that C-6, namely, the backbone structure of C-4–C-6 carbons in D-(6-^{13}C)glucose, is well preserved in the biosynthesis of curdlan. The dilution of labeling (19%), however, cannot be explained.

An experiment on labeling at C-2 gave unexpected results, that is, transfer of labeling from C-2 to C-1 and C-3. This transfer of labeling may however, be explained by the formation of fructose 6-phosphate from erythrose 4-phosphate and D-*threo*-pentulose (xylulose) 5-phosphate, resulting in transfer of labeling from C-2 to C-1, and by that from glyceraldehyde 3-phosphate and sedoheptulose 7-phosphate, resulting in transfer of labeling from C-2 to C-1 and C-3 in the pentose cycle, followed by the formation of curdlan. The percentage of curdlan formed from the latter fructose 6-phosphate was 11%, and the percentage formed from the former was 9%. Thus, 20% of the polymer is formed in this process.

The LR value (61%) at C-2 in curdlan obtained from D-(2-^{13}C)glucose agrees closely with the LR (57%) of C-1 in curdlan obtained from D-(1-^{13}C)glucose,

indicating direct polymerization of introduced glucose. Transfer of labeling from C-2 to C-5 was not observed, although Wolfrom et al.[12] reported the transfer in cotton cellulose prepared from D-[2-^{14}C]glucose. The cause for this lack of observation of transfer may be dilution of labeling, as already discussed. The decrease (39%) of LR at C-2 in curdlan obtained from D-(2-^{13}C)glucose may be attributed to isomerization of unlabeled D-glyceraldehyde 1-phosphate to dihydroxyacetone 1-phosphate, followed by neogenesis of glucose (8%), transfer of labeling from C-2 to C-1 and C-3 (20%) in the pentose cycle, and neogenesis of glucose from fragments formed in various pathways of glycolysis, followed by formation of curdlan (11%).

From these discussions, the biosynthesis of curdlan is tentatively explained by five routes: (*1*) direct synthesis of curdlan from labeled glucoses, including neogenesis of glucose and formation of curdlan without rearrangement of triose; (*2*) recombination of trioses to glucose with C-1 → C-3 rearrangement of phosphate groups; (*3*) recombination of isomerized (^{13}C-6 → ^{13}C-1) triose to glucose via the Embden–Meyerhof pathway; (*4*) formation of curdlan from reconstructed fructose 6-phosphate accompanying transfer of labeling (C-2 → C-1 and C-3) in the pentose cycle; and (*5*) neogenesis of glucose from fragments formed in various pathways of glycolysis. The percentages of routes (*1*) may be estimated as 60% from the LR of C-1 and C-2 in curdlans obtained from D-(1-^{13}C)glucose and D-(2-^{13}C)glucose, respectively. The percentages of route (*2*) is estimated as 3% from transfer of labeling from C-1 to C-3. The percentages of routes (*3*) and (*4*) are estimated as 8 and 20%, respectively, from transfer of labeling from C-6 to C-1 and that from C-2 to C-1 and C-3, as stated before.

Dilution of labeling (43%) at C-1 in curdlan obtained from D-(1-^{13}C)glucose is explained by mechanism *2* (3%), mechanism *3* (8%), mechanism *4* (20%), and mechanism *5* (11%). Details of mechanism *5* are unknown at present.

In conclusion, the biosynthesis of curdlan was interpreted as follows. Direct synthesis of the polymer from glucose constitutes the major pathway. In addition, C-1 → C-3 rearrangement and C-6 → C-1 isomerization in cleaved trioses formed via the Embden–Meyerhof pathway, and reconstruction of fructose 6-phosphate causing transfer of C-2 labeling to C-1 and C-3 in the pentose cycle, followed by neogenesis of glucose and formation of curdlan, occur to various extents. It should be stressed that the percentages of routes through which the polymer is formed may vary according to the culture conditions.

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