Natural Occurrence of Free Laminaribiose in the Seeds of Japanese Cycad*

(Studies on Some New Azoxyglycosides of *Cycas revoluta* Thunb. Part V)

Tomonori NAGAHAMA, Kotaro NISHIDA, and Tadao NUMATA

(*Laboratory of Biochemistry*)

In the previous reports on neocycasin A[1] and B[2], that is β-laminaribiosyl- and β-gentiobiosyl-oxyazoxymethane, a more convenient method for the inactivation of cycad emulsin by treatments with dilute acid was adopted in place of the traditional boiling procedures. It was, there, adverted that laminaribiose was detected in the extracts by paper chromatography. Under such an acidic condition, though the possibility of bringing about an acid reversion could be denied at that time, it was the question whether the appearance of laminaribiose, of which natural occurrence has not yet been reported, was caused by acid decomposition of neocycasin A, or not. In this paper, the facts of the latter case were confirmed with the results that it was isolated from the carefully boiled material without acid treating. The azoxyglycosides, too, were isolated in the same members as the ones by the acid treatment, of which pertinency was proved in reality.

Experimental and Results

Extraction and chromatographic fractionation of carbohydrates

The previous procedures[3] were some modified. Kernels, 6.37 kg, were thrown into boiling water few by few so as to continue the boiling incessantly. This process took some forty minutes. Boiled kernels were cooled rapidly, minced in a blender, and extracted six times repeatedly with every 5 *l* portions of 10 % ethanol. Combined extracts were concentrated at below 45° to syrup employing the Kestner's type apparatus. The treatments with methanol, lead acetate, and hydrogen sulfide were executed as ever. Thus obtained filtrates were concentrated in vacuo to 300 ml, of which 200 ml was chromatographed on a carbon column (dia. 5×40 cm; carbon, 250 g) by successive elutions with 0, 3, 5, 10, 15, 25, and 50% aqueous ethanol. The eluates were fractionated into 500 ml portions, of which carbohydrates were separately examined by paper chromatography, and were combined according to their constituents. As shown in Table 1, laminaribiose was slightly distributed together with cycasin in the 5% ethanolic eluates and mainly in the 10% ethanolic ones, of which components were complicated. Neocycasin A and B, and four more azoxyglycosides (spot $A_5 \sim A_8$) were eluted by ethanol of higher concentration.

* A part of this paper was presented preliminarily. (*J. Japan. Biochem. Soc.*, **31**, 428 (1959))

Table 1. Carbohydrates in Each Eluate from the Carbon Column found by Paper Chromatography.

H_2O		EtOH												
		3%	5	10	15		25			50				
No. 1	11	15	21	25	34	47	50	53	55	66	Rf		Colors revealed with reagents	
10	14	20	24	33	46	49	52	54	65	69			A.	R.
+	++	++	++	+	+	±					.53	cycasin	—	y
+	±	±									.44	fructose	pb	rb
++	±	±	±	±							.34	glucose	b	—
				+	+	+	++	+++	±		.30	neocycasin A	—	y
					+	±					.25	macrozamin	—	y
++	±	±	±								.23	sucrose	b	rb
					++	±					.18	neocycasin B	—	y
	-		+	++							.16	laminaribiose	b	—
									±		.14	A_5	—	y
							±	±	++	±	.10	A_6	—	y
									++		.007	A_7	—	y
				+	+						.055		b	rb
									+		.050		—	rb
				++	+	±	±				.045		gb	yb
									++		.030	A_8	—	y
					±	+	+	±	±		.015		gb	yb
							±	±	++	+	.00		p	y

Filter paper: Tôyô No. 2. Solvent: *n*-BuOH: AcOH: H_2O (4 : 1 : 1) Development: multiple ascending (2 runs). Reagents: R, resorcin–HCl EtOH soln.; A, aniline hydrogen phthalate BuOH soln. Colors: b, brown; y, yellow; r, reddish; g, greenish; p, pale.

Isolation and identification of laminaribiose

For the purpose of isolating laminaribiose from the fraction No. 25~33, the gradient elution technique[4] was employed, whereby the concentration of ethanol as eluting agent for the carbon column, was gradually increased from 5 % to 13.7 %. It showed, however, less effects on the separation of the co-existing components, and a preparative paper chromatography was further attempted. The materials were streaked along the starting line of paper sheets (60 cm in width×40 cm) and were ascended two times with a solvent mixture of *n*-butanol-acetic acid-water (4 : 1 : 1). With the aid of the guide strips, the bands containing laminaribiose were cut off, eluted with water, and concentrated in vacuo.

The resulting amorphous powder, 70 mg, was paper chromatographically pure and migrated at the same distance as it of authentic laminaribiose. Being incubated with cycad emulsin at pH 5.6 for sixteen hours, it was degraded completely into glucose. The powder,

30 mg, was acetylated as usual, the resulting material was recrystallized from ethanol, and 17 mg of acetate was obtained as fine needles, m. p. 158°~159° alone or on admixture with authentic octaacetyl-β-laminaribiose.

Anal. Found: C, 49.80 %; H, 5.96 %.
Calcd. for $C_{28}H_{38}O_{19}$: C, 49.56 %; H, 5.64 %.

Isolation of azoxyglycosides

The known azoxyglycosides were separated after usual manners from the above mentioned corresponding eluates. Their yields were as follows: cycasin from the fraction No. 11~24, 6.8 g; neocycasin A from No. 53~54, 2.1 g; neocycasin B from No. 34~46, 100 mg. A series of unknown glycosides, spot A_5~A_8, found in the higer numbered eluates is now further beeing separated. Among them the spot A_6 may be identical, in consideration of its Rf value, with neocycasin C[5] which was discovered during the course of the preceding study on enzymic transglycosylation.

Discussion

For the purpose of isolating the carbohydrates from natural sources intactly so as to not injure their native states, it would be necessary first to inactivate the enzymes which might degrade them, and next to choice the most mild procedures of extraction as possible. Being aimed at a certification of laminaribiose in cycad seeds under especial attentions to these points, here presented data can be said to give the evidence for the actual existence of this sugar in nature. Although the yield is very low, it might be due to the facts that this sugar was so difficult to freed from the co-existing oligosaccharides that several methods had to be successively tried, and it does not mean, of course, the natural content in the seeds.

Anyway, it is interesting that a free sugar, which has the β-1, 3 linkage of little known heretofore in nature, was proved together with azoxyglycosides in which the very sugar is composed. Furthermore, the proof for the same carbohydrate pattern in the extracts obtained by here adopted method as in that by acid treatment, makes the latter method reliable along with its conveniences.

Summary

An extraction of carbohydrates occurring in the seeds of Japanese cycad was performed under the deliberated conditions which did not caused their decomposition.

After chromatographical procedures using carbon columns and paper sheets, laminaribiose was isolated and identified.

The azoxyglycosides, which were isolated by the convenient acid treatment reported previously, was also obtained preparatively. Accordingly, it was confirmed that laminaribiose or these glycosides should be ones occurring naturally.

Acknowledgment: The authors wish express their thanks to Prof. T. Mitsui, Faculty of Agriculture, Kyoto University for his favours in performing the elementary analysis.

References

(1) K. NISHIDA, A. KOBAYASHI, T. NAGAHAMA, and T. NUMATA, *Bull. Agr. Chem. Soc. Japan*, **23**, 460 (1959)
(2) T. NAGAHAMA, T. NUMATA, and K. NISHIDA, *ibid.*, **23**, 556 (1959)
(3) K. NISHIDA, A. KOBAYASHI, and T. NAGAHAMA, *ibid.*, **19**, 77 (1955)
(4) R. M. BOCK and NAN-SING LING, *Anal. Chem.*, **26**, 1543 (1954)
(5) T. NAGAHAMA, K. NISHIDA, and T. NUMATA, *Bull. Agr. Chem. Soc. Japan*, contributed