Introduction of Fatty Acids into Low-Lipid Starches and Their Nägeli Amylodextrins

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ABSTRACT

Fatty acids (FA) were artificially introduced into 14 kinds of low-lipid starches and their Nägeli amylodextrins (NA). The introduced palmitic acid (PA) and linoleic acid (LA) in starches amounted to 65.1–287.2 and 33.5–132.9 mg/100 g of starch, respectively, which was generally related to the granular size of the starches. The introduction of PA interfered in the color development of the starches with iodine. In the case of NA, PA could be also introduced into the NA to some extent (19.6–91.7 mg/100 g of NA), which indicated that the NA still had capacity for introducing PA. On the basis of the amounts of introduced PA in the whole granule and in the NA, it was considered that the quantities in the whole granules might depend on the intact acid-soluble part. Statistical analysis of the chemical structures of NA showed that the quantity of introduced PA in NA was positively correlated to the limit of β-amylolysis and negatively to the number of branched points. This result indicated that the larger the amount of PA introduced in NA, the more linear the molecules constituting NA.

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Starch granules naturally contain bound lipids, which are hardly extractable with common fat solvents such as diethyl ether and chloroform. Bound lipids are abundant in cereal starches (0.5–1.0%) except for waxy type (Morrison 1988), while they are relatively low in abundance in root and tuber starches, such as potato, cassava, and sweet potato starches (Fujimoto et al 1972). As is generally known, lipids (including surfactant) affect the physicochemical properties of starches. Also, the bound lipids in starch granules may contribute to the characteristic pasting feature of starches (Tester and Morrison 1990a,b, 1992, 1993; Morrison et al 1993a; Tester et al 1993).

On studies of artificial introduction of fatty acids (FA) into starches (Kitahara et al 1993), in which differences in the quantities of FA introduced into the starches were found, we used three tuberous starches without defatting because the starches were originally low in lipid content, fearing that undesirable denaturation of the granular structure might be caused concomitantly by defatting of the original bound lipids with hot aqueous alcohol.

In the present study, we selected 14 kinds of low-lipid starches from commercial products and wild plants grown in southern Japan, and examined the characteristics of introducing FA into the starches and their Nägeli amylodextrins (NA). The results showed useful information concerning not only the nature of the starch-lipid relationship but also the granular structure of starch.

MATERIALS AND METHODS

Starches and Reagents

Potato, sweet potato, cassava (tapioca), and *kuzu* (*Pueraria hirsuta* Matsum) starches were commercial products and purchased from Katsuo-shoten (Kagoshima), Ei Agricultural Cooperatives (Kagoshima), Katsuo-shoten and Hirohachido-shouten (Kagoshima), respectively. Arrowroot (*Maranta arundinacea* L.) starch was kindly donated from L. S. Palomar, Visayas State College of Agriculture, Philippines. Normal and waxy corn starches were obtained from Nihon Shokuhin Kako Co., Ltd. (Tokyo). These commercial products were high-grade, but not specially processed.

Other starches were prepared from wild plants grown in Kagoshima Prefecture, Japan: Rhizomes of *shokuyo-kanna* (*Canna edulis* Ker.), *gajutsu* (*Curcuma zedoaria* Roscoe), and *ukon* (*Curcuma domestica* Valeton), bulb of *teppo-yuri* (*Lilium longiflorum* Thunb.), tuberous root of *karasu-uri* (*Trichosanthes cucumeroides* Maxim.), and stem of *sotetsu* (*Cycas revoluta* Thunb.). They were mashed with deionized water and then passed through a 200-mesh screen. The starches were repeatedly suspended and settled in deionized water to be purified. In the case of banana (*Musa paradisiaca* Lin. var. *sapientum* O. Kuntze) and *basho* (*Musa basjoo* Sieb. et Zucc.), the tubers were cubed, soaked in 0.25% sodium hydrogen sulfite overnight, and then mashed with 0.2% sodium hydroxide to prevent the starches from browning and adsorbing viscous material (Fujimoto et al 1985). The starches were thoroughly washed with deionized water. All starch samples were washed out with diethyl ether for 24 hr in a Soxhlet extractor to remove lipids adsorbed on the surface of granules.

If necessary, defatted starch sample was prepared by extraction with hot 85% methanol (1 hr × 3 times). NA was prepared by leaching starch with 15% sulfuric acid (w/w) at 35°C (shaken by hand once a day) for 25–30 days until corrosion of the starch, which was detected by the phenol-sulfuric acid method (Dubois et al 1956), leveled off. Microscopic observation confirmed that the granules mostly maintained their shape and also that the peak intensity on x-ray diffractograms of all starches was increased by the acid treatment. The NA were also washed out with diethyl ether, as in the case of native starch.

All reagents and solvents, unless otherwise specified, were obtained from Wako Chemical Industries (Osaka) and were of reagent grade.

General Properties of Starches

Granular sizes of starch were measured on the photomicrograph, and their average sizes were calculated from the distributions of at least 500 granules. Nitrogen was determined by the semi-micro Kjeldahl method, and crude protein content was represented as N × 6.25. Phosphate attached to glucosyl residues was converted into inorganic phosphate by wet ashing of defatted starch (40 mg, dry matter) with 60% perchloric acid (1 ml) and with the aid of four drops of nitric acid. The phosphate was determined by the method of Fiske and Subbarow (1925). The apparent amylose content was calculated from the respective blue value of defatted starch sample (McCready and Hassid 1943) on the basis of those of amylose (1.47) and amylopectin (0.21) from

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sweet potato starch; the blue value was determined at concentrations of 40 μg of starch, 80 μg of iodine, and 800 μg of potassium iodide per milliliter. The coefficients of variation of the respective triplicate experiments were <1%. X-ray diffractograms were obtained under the conditions previously reported (Kitahara et al 1993).

Free fatty acids (FFA) in starch granules were determined using an NEFA-C test kit (Wako, Osaka) as previously reported (Kitahara et al 1994). The amount of bound lipids in starch was simply evaluated as total FFA after degradation of starch with 0.1*N* hydrochloric acid. Starch (300 mg, dry matter) was hydrolyzed by 0.1*N* hydrochloric acid (2 ml) for 2 hr at 100°C. After neutralization of the hydrolysate with 1*N* sodium hydroxide, the solution was increased to 4 ml with 1-propanol, and the FFA in the aliquot solution were also determined as palmitic acid (PA) using an NEFA-C test kit. The coefficient of variation of the method was <3%.

Introduction of FA into Starches

Starch was mixed with 1% PA or 1% LA (>99 %, Nacalai Tesque Inc., Kyoto) in 85% methanol for 3 hr at 35°C with gentle stirring (Kitahara et al 1993). The treated starch was washed with diethyl ether for 24 hr in a Soxhlet extractor to remove extraneous FA. The amount of introduced FA was determined using an NEFA-C test kit; naturally occurring bound FFA was subtracted from the amount. The coefficient of variation of this triplicate experiment was <5%.

Chemical Structures of NA

Debranching of NA solution was done by isoamylase (*Pseudomonas* sp.) (Nacalai Tesque Inc.) in 60 m*M* acetate buffer (pH 3.5) at 45°C for 12 hr. The chain length distribution of debranched starch was determined using high-performance liquid chromatography (Tosoh Co. Ltd., Tokyo) (Kitahara 1994). Two sequentially linked columns of Superose 12 (1 × 30 cm, Pharmacia, Uppsala, Sweden) and Sephadex G25 (1 × 30 cm, Pharmacia) were calibrated with synthesized linear amyloses (DP = 1,815, 728, 438, 165, 63, Azinoki, Aichi), AMYLOSE EX-1 (DP = 17, Hayashibara Biochemical Laboratories Inc., Okayama) and maltopentaose (Nacalai Tesque Inc.). The analysis was made in duplicate determinations.

The average degree of polymerization (dp) was determined by the method of Hizukuri et al (1981). The average chain length (cl) was expressed as the dp of debranched NA after the isoamylase treatment. The limit of β-amylolysis was determined by the method of Hizukuri et al (1981). The average number of branched points (nb) was calculated from the equation: (dp/cl) – 1. The coefficients of variation of these triplicate experiments were <1%.

Other Experiments

Starch granules (300 mg, dry matter) were suspended in 0.02% iodine solution including 0.2% potassium iodide (3 ml). After filtration, chromaticity values (L^*, a^*, b^*) of the stained starch were measured using a Minolta colorimeter CR 200. The values are expressed as the average of five determinations. Hue difference (ΔH^*) between the colors of native and FA-introduced starches was calculated as:

$$\Delta H^* = \{(\Delta a^*)^2 + (\Delta b^*)^2 - (\Delta C^*)^2\}^{1/2}$$

$$\Delta C^* = \{(a^*_{\text{introduced}})^2 + (b^*_{\text{introduced}})^2\}^{1/2} - \{(a^*_{\text{native}})^2 + (b^*_{\text{native}})^2\}^{1/2}$$

All statistical analyses were done by using SPSS Education Version 4.0 on a Macintosh computer.

RESULTS AND DISCUSSION

General Properties of Starches

The general properties of the starches used here are shown in Table I. The average granule size of the starches ranged from 3.8 μm to 45.8 μm, and are listed in the order of decreasing size. All starches had normal size distributions, although the starches with relatively large average granular size, showed broader size distributions. The crude protein content of all samples was <0.1%, which indicated successful isolation of the starches. Among the starches from wild plants, *ukon* and *gajutsu* starches were richer in attached phosphate content than was potato starch, and their apparent amylose contents (27.0 and 32.5%, respectively) were relatively high compared to other starches. The x-ray diffraction patterns of most starches were B or C type, which were common to those of tuber and root starches, except for A type of cassava starch.

In the previous study, Kitahara et al (1993) found that a treatment of starch with 0.1*N* hydrochloric acid in boiling water released FA from acyl lipids in the starch during the treatment. The amount of total bound lipid, therefore, was estimated as total

TABLE I
General Properties of Starches

Starch[a]	X-ray Diffractogram	Average Size[b] (μm)	Crude Protein[c] (%)	Phosphorus[d] (%)	Apparent Amylose[e] (%)	Bound Lipid[f] (%)
Shokuyo-kanna	B	45.8	0.08	0.04	26	0.02
Potato	B	40.0	0.05	0.09	19	0.01
Banana	B	37.5	0.09	0.03	19	0.02
Basho	B	32.4	0.06	0.03	21	0.03
Teppo-yuri	B	32.3	0.03	0.01	25	0.05
Gajutsu	B	25.6	0.04	0.22	27	0.03
Ukon	B	20.3	0.05	0.24	33	0.02
Cassava	A	11.4	0.03	0.01	13	0.04
Sotetsu	C	10.0	0.08	<0.01	19	0.02
Arrowroot	C	10.0	0.05	0.02	16	0.03
Sweet potato	C	9.8	0.08	0.02	16	0.05
Kuzu	C	8.5	0.07	0.02	17	0.12
Udo	B	4.0	0.06	0.02	11	0.04
Karasu-uri	B	3.8	0.01	0.11	21	0.05

[a] Respective scientific names are shown in the text.
[b] Calculated from size distribution on microscopic observation.
[c] Determined by the Kjeldahl method.
[d] Determined by the Fiske-Subbarow method after $HClO_4$-degradation of starch.
[e] Calculated from the respective blue value on the basis of those of amylose (1.47) and amylopectin (0.21) from sweet potato.
[f] Determined using NEFA-C Test Wako kit after HCl-hydrolysis of starch.

FFA obtained by the acid hydrolysis method. As also seen in Table I, the contents in the starches were 0.01–0.05 % except for *kuzu* starch (0.12%), and naturally occurring FFA comprised more than half of the amount (data not shown). Thus, it was considered that bound lipids in these starches were much less than those of cereal starches (0.5–1.0 %, Morrison 1988).

Characteristics of Introduced FA in Starches

Among the FA composition of naturally occurring lipids in starch, PA and LA are the main FA (Morrison 1988). The PA and LA were artificially introduced into starches under more moderate conditions in 85% methanol at 35°C rather than at higher temperatures (Lehrman 1942, Fujii and Oba 1962, Fujimoto et al 1972) or in FA sodium salt solution (slightly alkaline solution) (Ohashi et al 1980) to minimize denaturation of granular structure.

Figure 1 shows the amounts of introduced PA and LA in the present study. The respective amount of PA introduced into starch (65.1–287.2 mg/100 g of starch) was larger than that of LA (33.5–132.9 mg/100 g of starch), which agreed with the previous result that more saturated FA was introduced into starch than unsaturated FA (Kitahara et al 1993). It was also observed that the smaller the granular size of the starches, the larger the amount of introduced FA. This implied that the amount introduced was closely correlated to the total surface area of the granules per weight. The observation was evident for LA ($Y = 0.02X^2 - 2.78X + 122.47$, $r = 0.874$). In the case of banana and *ukon* starches, however, relatively high amounts of PA were introduced into the starches in contrast to the amounts of LA. Microscopic observation of both starches showed thin and flat granular shapes, but no pores or pits were found. A further study on their granular structure is required.

The reaction of starch granules with iodine results in a purple coloration attributed to its inclusion in the helical lumen of starch molecules. Colors of iodine-stained native and PA-introduced starch were determined using a hue-difference colorimeter. The positive values of a^* and b^* indicated red and yellow colors respectively; their negative values are green and blue respectively. As seen in Table II, the values of both a^* and b^* became positive following the introduction of PA, which indicated change in color from a bluish purple to a pale reddish one. The results indicate that the introduced PA interfered with color development, and suggest that the FA molecules might exist in the helical cavity of starch molecules in a manner similar to that of iodine. However, only *teppo-yuri* starch showed no change in the color as seen in the value of hue-difference (ΔH^*) indicating the extent of color change. The reason for this behavior is unknown.

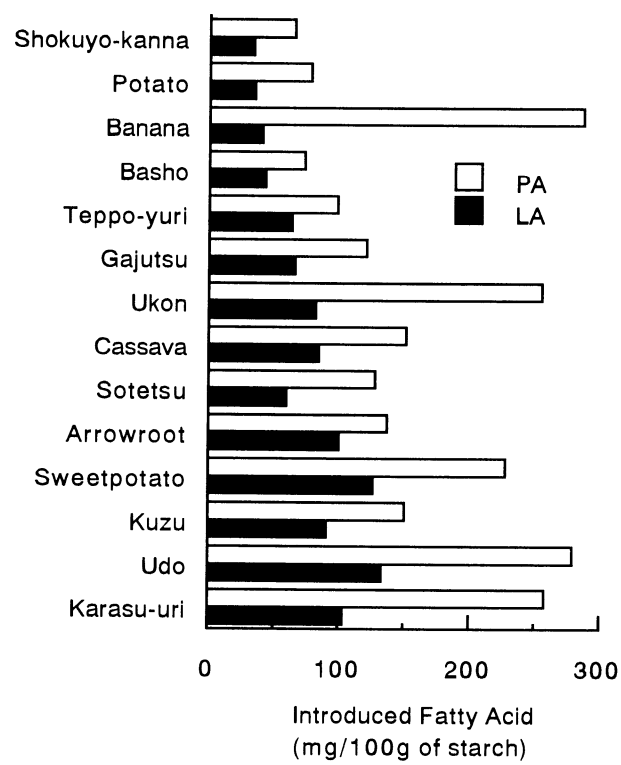

Fig. 1. Introduction of palmitic acid (PA) and linoleic acid (LA) into starches.

Introduction of PA into NA

NA is an acid-resistant part of starch granules, which was considered to be a highly crystalline residue, mainly double helices from clusters of amylopectin, after eroding away amorphous parts (Kainuma and French 1971, Umeki and Kainuma 1981, French 1984). PA was also introduced into the NA, and the distribution of PA between the acid-resistant part and acid-soluble part was estimated from amounts of introduced PA in the whole granule and in the NA as:

(introduced PA in acid-resistant part; mg/100 g of starch) = (introduced PA in NA; mg/100g of NA) × (yield of NA; %/100)

(introduced PA in acid-soluble part) = (introduced PA in whole granule) – (introduced PA in acid-resistant part)

It was emphasized that the introduced PA was not extractable with diethyl ether. The results are shown in Table III. The introduced PA in NA amounted to 19.6–91.7 mg/100 g of NA. The amount of introduced PA in the acid-soluble part that was estimated to occupy approximately half of the granules from the yield of NA was calculated to be 24.9–267.4 mg/100 g of starch. The calculated amounts of potato, *teppo-yuri,* and *gajutsu* were in agreement with a previous report (Kitahara et al 1993). The quantities of introduced FA in the whole starches that related to the granular size of starches were more reflected by the quantities of introduced FA in the acid-soluble part than in the acid-resistant part. It was, therefore, considered that the quantities of PA introduced in the whole starches were dependent on that in the intact acid-soluble part.

Another interesting aspect of this experiment was that some of the PA could be introduced into NA, i.e., NA still had a little

TABLE II

Chromaticity Values and Hue Difference (ΔH^*) of Iodine Coloration of Native and Palmitic Acid (PA)-Introduced Starches

	Native Starch		PA-Introduced Starch		
	a^{*a}	b^{*b}	a^{*a}	b^{*b}	ΔH^{*c}
Shokuyo-kanna	+5.72	−8.42	+6.43	−7.65	1.03
Potato	+5.75	−12.36	+6.50	−8.68	2.52
Banana	+5.02	−7.05	+5.99	−3.82	3.00
Basho	+5.27	−8.82	+6.47	−7.35	1.84
Teppo-yuri	+7.75	−8.32	+7.41	−8.07	0.08
Gajutsu	+3.92	−6.53	+7.73	−6.15	3.09
Ukon	+4.74	−8.12	+8.05	−6.11	3.81
Cassava	+5.21	−10.42	+8.76	−4.94	6.33
Sotetsu	+5.55	−9.83	+6.24	−6.33	2.64
Arrowroot	+6.87	−10.05	+9.49	−5.38	5.20
Sweet potato	+6.60	−10.25	+9.31	−4.88	5.78
Kuzu	+6.76	−7.53	+7.64	−5.00	2.49
Udo	+8.50	−11.11	+9.39	−8.27	2.58
Karasu-uri	+8.76	−11.64	+10.26	−8.26	3.42

[a] Green (-) to red (+).
[b] Blue (-) to yellow (+).
[c] Calculated from the equations shown in the text.

capacity for incorporating PA even though all NA were prepared until corrosion of the starch with the acid leveled off. As seen in Table IV, the dp of NA ranged from 17.1 to 21.1, which were comparable to that (20.0) of waxy corn (Kikumoto and French 1983). In the common starches of potato, the cl were slightly shorter than the respective external chains of amylopectin (Hizukuri 1985). From fact that the cl were smaller than the dp, that the NA were not completely hydrolyzed by β-amylase, and that nb were <1, it was confirmed that all NA determined also consisted of both linear and branched dextrins (French 1984). Table V shows a correlation matrix among variables from chemical structures of NA and the amount of introduced PA in NA. Among the chemical structures of NA, significant correlations were found: 1) the dp was negatively correlated to the limit of β-amylolysis and positively to the nb; 2) the cl and the cl of each gel-permeation chromatogram (GPC) peak of debranched NA were positively correlated to one another and negatively to the nb; and 3) the limit of β-amylolysis was negatively correlated to the nb. These results suggested that NA of large dp had more branching points, thus the limit of β-amylolysis was lowered. It was also suggested that the larger the cl or the cl of GPC peak, the more linear the molecules.

The amount of introduced PA in NA was positively correlated to the limit of β-amylolysis and negatively to the nb, which indicated that the amount of introduced PA increased with that of linear molecules constituting NA.

Morrison et al (1993b) reported that lintner residue from non-waxy barley starch consisted of some partially hydrolyzed fragments. Among them, the fragment (cl = 46) on GPC was derived from lipid-free amyloses retrograding into double helices, and the longer chains (cl = 77, 120–130) were from lipid-complexed amyloses. In our study, GPC analysis revealed that debranched NA from low-lipid starches had a single peak at cl = 18–30 with a shoulder at cl = 50–60 that might correspond to the fragment from lipid-free amyloses; there was no further longer chain (data not shown). Although a mode of action of sulfuric acid was slightly different from that of hydrochloric acid (Hizukuri et al 1972), the chromatograms obtained here appear to be consistent with their concept. They also suggested the possibility that the V-type conformation was resistant to acid hydrolysis as well as double helices. As seen in Table V, the amylose content was closely correlated to the cl and the cl of a peak of NA. It has been reported that amyloses are interspersed among amylopectin molecules in starch granules (Jane et al 1992, Kasemusuwan and Jane 1994). It was, therefore, considered that a part of the linear molecules in NA might be derived from amylose. As the amylose content used here was estimated from the blue value of starches, it is necessary to determine fine structures of amylose and amylopectin of the starches, including their relation to the structure of acid-resistant residue. However, the existence of some space (presumably helical cavity) in NA was confirmed by this experiment using PA as a probe.

Table VI shows results from normal and waxy corn starches. Contrary to the result from lintnerized rice starch (Maniñgat and Juliano 1979) or barley starch (Morrison et al 1993b), more than 90% of bound FFA was liberated from the granules by the acid treatment. The loss of bound FFA in the corn starch was also observed in the case of crude glucoamylase digestion (Kitahara et al 1994). The reasons for this are currently under study. When PA was introduced into the NA, some PA could also be introduced into them, even waxy ones. Amylose of the waxy corn starch used here was a trace amount estimated from GPC analysis of the debranched starch. It was, therefore, considered that the some space in NA might be derived from not only amylose but also amylopectin.

CONCLUSION

FA were introduced into 14 kinds of low-lipid starches, and characteristics of the introduction of FA into the starches and their NA were investigated. It was found that the amount of introduced FA into starch was primarily related to the average granular size of the starches and might depend on the intact structure of the acid-soluble part of granules. Because introduced PA are firmly retained in starch (unextractable with diethyl ether) and interfere with the iodine coloration of starch, it was considered that the FA might be introduced into helical cavities in starch.

When FA are used as a probe to search for the cavity, NA, even though that is from waxy corn, still have some capacity for introducing FA. The NA used in this study were stained by iodine, although the color tones of iodine staining were different from each other. We consider that the cavity in NA might be derived from not only amylose but also amylopectin, because of the fact that those of waxy-corn starch were also stained by iodine.

TABLE III
Introduction of Palmitic Acid (PA) into Nägeli Amylodextrins (NA) and Possible Amounts in Starch Granules

	NA	PA[b]	
	Yield (%)	Introduced PA[a]	Acid-Resistant[c]	Acid-Soluble[c]
Shokuyo-kanna	59.6	67.4	40.2	24.9
Potato	53.1	50.7	26.9	51.0
Banana	53.7	36.9	19.8	267.4
Basho	56.7	27.2	15.4	57.7
Teppo-yuri	53.9	91.7	49.4	48.7
Gajutsu	55.7	85.3	46.8	73.8
Ukon	54.9	38.6	21.1	234.0
Cassava	50.5	19.6	9.9	141.4
Sotetsu	44.0	56.8	19.4	108.2
Arrowroot	51.7	31.7	16.4	120.2
Sweet potato	55.1	20.1	11.1	216.4
Kuzu	54.2	27.5	14.9	135.6
Udo	31.2	46.2	18.1	261.2
Karasu-uri	52.7	57.1	27.7	230.3

[a] mg/100 g of NA.
[b] Amounts of introduced PA in acid-resistant and acid-soluble portions were calculated from equations shown in the text.
[c] mg/100 g of starch.

TABLE IV
Chemical Structures of Nägeli Amylodextrins

	DP[a]	CL[b]	Peak CL[c]	β-Amylolysis[d] (%)	BP[e]
Shokuyo-kanna	17.1	16.2	23	90.5	0.05
Potato	19.3	15.6	22	86.3	0.24
Banana	18.1	15.8	22	82.4	0.15
Basho	19.1	16.1	22	80.0	0.17
Teppo-yuri	17.6	16.0	23	92.4	0.10
Gajutsu	19.5	17.0	26	82.8	0.15
Ukon	21.1	17.8	30	78.0	0.19
Cassava	20.8	15.0	18	76.8	0.39
Sotetsu	18.8	15.0	19	85.4	0.25
Arrowroot	19.6	15.6	20	78.6	0.26
Sweet potato	19.0	15.1	20	84.7	0.26
Kuzu	20.5	15.3	20	79.2	0.34
Udo	20.7	15.0	19	81.2	0.38
Karasu-uri	19.5	16.1	23	79.3	0.21

[a] Average degree of polymerization.
[b] Average chain length.
[c] Chain length of peak GPC of debranched NA.
[d] Limit of β-amylolysis.
[e] Average number of branched points.

TABLE V
Correlation Coefficients Between Analytical Data

	Nägeli Amylodextrins					
	DP[a]	CL[b]	Peak CL[c]	β-Amylolysis[d]	NB[e]	Introduced Palmitic Acid
Amylose content	−0.239	+0.918**[f]	+0.926**	+0.268	−0.764**	+0.525
DP		+0.019	+0.009	−0.825**	+0.777**	−0.500
CL			+0.980**	−0.037	−0.611*	+0.371
Peak CL				+0.042	−0.603*	+0.394
β-Amylolysis					−0.617*	+0.675**
NB						−0.612*

[a] Average degree of polymerization.
[b] Average chain length.
[c] Chain length of peak GPC of debranched NA.
[d] Limit of β-amylolysis.
[e] Average number of branched points.
[f] * = P <0.05, ** = P <0.01.

TABLE VI
Introduction of Palmitic Acid (PA) into Nägeli Amylodextrins (NA) of Normal and Waxy Corn Starches

	Native Starch (mg/100 g)	NA (mg/100 g)		
	Bound FFA[a]	Yield (%)	Bound FFA	Introduced PA
Normal corn	505.5	49.7	20.4	58.2
Waxy corn	41.8	39.2	2.9	19.1

[a] Free fatty acids.

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