

Microdetermination of Surface Proteins of Wheat Starch Granules by Dye Binding

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The presence of surface protein on wheat starch granules was reported by Lowy et al (1981), Seguchi (1984a–c), Malouf et al (1992), and Baldwin et al (1997). The protein accompanying wheat starch is generally assayed as $N \times 5.7$ (Smith 1964), and it occurs not only as surface protein but also as intragranular protein. It was reported that chlorination of wheat flour (Seguchi 1984a,b; 1985) and heat-treatment at 120°C for 2 hr (Seguchi 1984c, 1986a) changed the nature of the wheat starch granules' surface from hydrophilic to hydrophobic, possibly due to a change in the wheat starch granules' surface protein. The hydrophobicity of the wheat starch granule surface by chlorination or heat treatment was related to improvement in pancake texture such as springiness and gumminess in the mouth (Seguchi and Matsuki 1977, Seguchi 1990).

To clarify the nature and role of the wheat starch granule surface protein, a microdetermination method of the level of surface protein is needed. Nakao et al (1973) reported a method for protein determination using dye binding of amido black 10B dye. The binding of this dye is not easily inhibited by other materials. Kaplan and Pedersen (1985) reported this dye method could be used in the presence of high levels of lipids. Yokotsuka et al (1978) determined the protein content in fruit juice, vegetable juice, and wine using the amido dye. In this study, a modified dye method was designed, and the level of surface protein on wheat starch granules was determined using the method.

MATERIALS AND METHODS

Materials

A wheat starch sample was obtained from soft wheat flour using an acetic acid (pH 3.5) fractionation technique (Sollars 1958, Seguchi and Matsuki 1977). An air-classified wheat starch sample was donated by Okumoto Flour Milling Co., Japan, and a commercially purchased wheat starch sample was also used. The membrane filter used was plain-surfaced cellulose nitrate with a pore size of 0.3 μm and diameter of 90 mm (Type TM-3, Toyo Roshi Co., Tokyo, Japan). Amido black 10B was purchased from Nacalai Tesque, Kyoto, Japan. The elemental formula for amido black 10B is $\text{C}_{22}\text{H}_{14}\text{N}_6\text{O}_9\text{S}_2\text{Na}_2$ (mol. wt. = 616.50) and its N content is 13.6%. Its molar extinction coefficient at 630 nm in 25 mM sodium hydroxide containing 50 vol% ethanol and 0.05 wt% SDS was 1.77×10^4 . All other reagents were high grade and purchased from commercial sources.

Determining Starch Surface Protein by Dye-Binding Method

All experiments were performed at room temperature and were replicated at least three times. Amido black 10B (1 g) was stirred in 100 mL of *n*-propanol, acetic acid, and water at 3:1:6 (v/v) and the mixture was filtered through a membrane filter by suction just

before use. A membrane filter 10 mm in diameter was cut down from one 90 mm in diameter. A starch sample (20–80 mg) was suspended in water (0.1 mL) and mixed with the same volume of the amido black 10B solution. The mixture was left standing for 20 min. After centrifugation at $600 \times g$ for 5 min, the supernatant fraction was discarded. The pellet was then suspended in 10 mL of 90% methanol solution containing 2% acetic acid and was centrifuged at $600 \times g$ for 5 min. This washing process was repeated three times. Using water (10 mL) under the same washing conditions, the process was repeated 10 times. The washed starch pellet was suspended in 10 mL of 50 mM sodium hydroxide solution containing 0.1% SDS, shaken vigorously for 2 hr, and centrifuged at $500 \times g$ for 5 min. The resulting supernatant was diluted with the same volume of absolute ethanol. Optical density was measured at 630 nm ($\text{OD}_{630\text{nm}}$) using a spectrophotometer (Shimadzu Spectronic 20).

Standard Curve of Bovine Serum Albumin

Bovine serum albumin (BSA) was used as a standard protein. A standard curve was prepared using the method of Yokotsuka et al (1978). BSA solution (100 mL, 67–333 μg of protein) was mixed with 100 μL of 1% amido black 10B solution in a 10-mL conical tube. The mixture was shaken vigorously, left stand for 20 min, and then filtered on the membrane filter by suction. The precipitate on the membrane filter was washed with 2.5 mL of 30% propanol containing 10% acetic acid and then washed three times with 100 mL of 90% methanol containing 2% acetic acid. The background reading of the blank test indicated ≈ 0 at $\text{OD}_{630\text{nm}}$ after the third washing. The washed membrane filter was shaken for 20 min in 10 mL of 25 mM sodium hydroxide solution containing 0.05 wt% SDS and 50 vol% ethanol and the $\text{OD}_{630\text{nm}}$ of the extract was measured.

Removing Wheat Starch Granule Surface Protein

Wheat starch granules (10 g) were placed in 200 mL of 1% SDS containing 1% 2-mercaptoethanol (2-ME) in water, and the suspension was warmed to 37°C and kept for 2 hr with occasional shaking. After centrifugation at $500 \times g$ for 10 min, the sedimented starch was extracted at least four times with fresh SDS and 2-ME. The protein contents ($N \times 5.7$) of the starch granules before and after extraction were determined by the Kjeldahl method.

Staining Washed Starch Granules with Amido Black 10B

A sample (0.5 g) of wheat starch that had been washed with SDS and 2-ME was further washed with a large amount of water and stained with amido black 10B (Seguchi 1986b). The stained starch sample was put into a petri dish (3 cm diameter), dried at room temperature, and photographed.

RESULTS AND DISCUSSION

Determining Concentration of Amido Black 10B

The concentration of amido black 10B solution was raised from 0.25 to 1.00%, and the dye solutions were used to stain wheat starch granules suspended in an equal volume of water. After removal of excess dye from the granules by washing, the dye bound to the

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stained starch granules was released with ethanolic SDS and alkali. The OD_{630nm} value of bound dye showed a constant value when the concentration of the amido black 10B solution was >0.75% (Fig. 1A). In the remaining experiments, 1.00% of amido black 10B solution was used to dye the starch.

Washing Stained Starch Granules with Water

After triple washing with 10 mL of 90% methanol solution containing 2% acetic acid, the level of dye associated with wheat starch decreased (data not shown). Subsequent washing of the dyed starch 10 times in water resulted in an asymptotic value of bound dye (Fig. 1B). The protein content of an unstained starch sample washed with 90% methanol and 2% acetic acid followed by washing 10 times with water was equal (101.1%) to that of the unstained and unwashed sample. Those results indicate that washing of the dyed wheat starch did not remove the protein at the starch granule surface but only removed unbound dye.

TABLE I
Effects of Concentration of Sodium Hydroxide and 50% Ethanol on the Extraction of Dye

Treatments	OD _{630nm} ^a
25 mM NaOH containing 0.1% SDS	0.096
50 mM NaOH containing 0.1% SDS	0.105
75 mM NaOH containing 0.1% SDS	0.092
100 mM NaOH containing 0.1% SDS	0.230
50 mM NaOH containing 0.1% SDS and 50% ethanol	0.023

^a Means of determinations performed at least three times. Standard deviation ± 0.01.

TABLE II
Surface and Total Protein in Wheat Starch Granules^a

Source of Wheat Starch	Starch Granule Surface Proteins by Dye Method (%)	Whole Starch Proteins by N × 5.7 (%)	% Surface Protein
Fractionated using acetic acid	0.18	0.23	78.3
Air-classified	0.18	0.21	85.7
Commercial source	0.16	0.27	59.3

^a Means of determinations performed in at least three times. Standard deviation of the dye method and N × 5.7 were 0.01 and 0.05, respectively.

Release of Dye Bound to Stained Starch Granules

The amido black 10B dye bound to the stained starch was completely extracted using weak alkali as judged visually. As indicated in Table I, the concentration of sodium hydroxide solution containing 0.1% SDS was increased from 25 mM to 100 mM. The OD_{630nm} value of the extracted solution increased when the concentration of the 25 mM sodium hydroxide reached 50 mM. However, when 100 mM sodium hydroxide was used, the starch granules were gelatinized, and OD_{630nm} could not be measured because of the turbidity of the starch solution. When 75 mM sodium hydroxide was used, slight swelling of the starch granules was observed. Because the starch did not swell and gelatinize in 50 mM sodium hydroxide, and a nonswollen opaque starch granule was obtained after extraction using the alkali solution, 50 mM sodium hydroxide containing 0.1% SDS was used to extract the bound dye. Yokotsuka et al (1978) used 25 mM sodium hydroxide solution containing 50% ethanol for the dye extraction, and they reported that ethanol was needed to extract the dye. For stained wheat starch granules, 50 mM sodium hydroxide solution containing 0.1% SDS and 50% ethanol extracted the bound dye to some extent but not completely (Table I). This indicated that 50% ethanol inhibited the extraction of bound dye from starch granules.

In our laboratory, a BSA standard curve was prepared using the method of Yokotsuka et al (1978) and included the use of ethanol. To use the standard curve according to Yokotsuka et al, ethanol was added to the extracted dye solution just before the determination of OD_{630nm}. The resulting final concentrations at the point of reading OD_{630nm} were 25 mM sodium hydroxide, 0.05% SDS, and

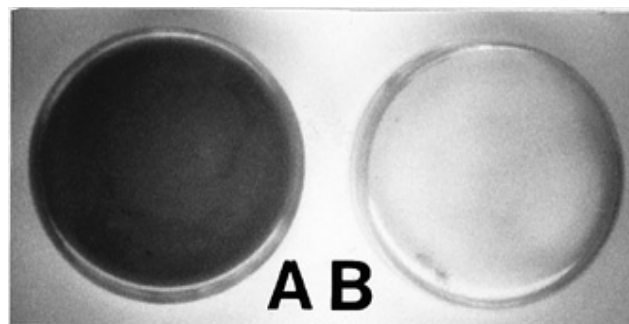


Fig. 2. Wheat starch granules stained with amido black 10B, washed with water, and dried in a petri dish; before (A) and after (B) extraction.

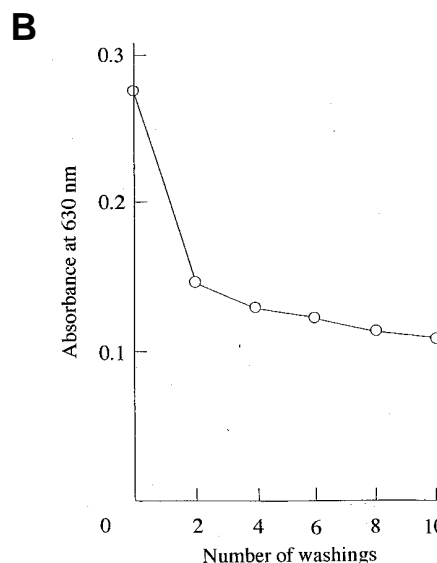
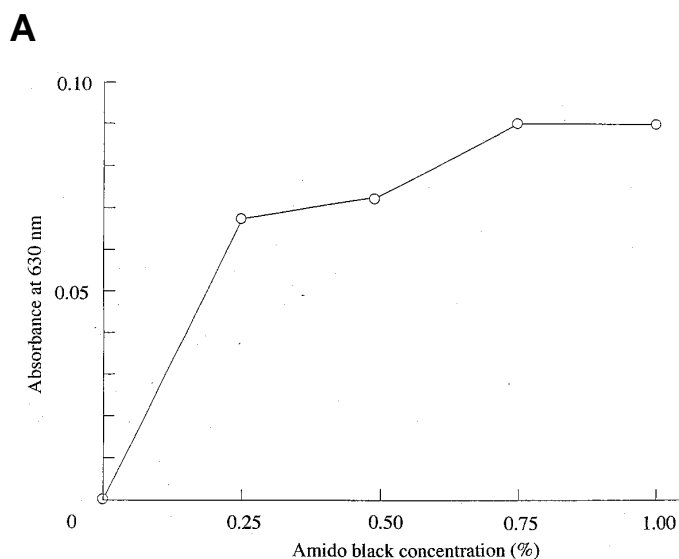


Fig. 1. A, Effect of dye concentration on the staining of wheat starch granules. Standard deviation = 0.003. **B,** Effect of the number of washings with water on the level of bound dye. Standard deviation = 0.004. Data points represent mean of duplicate determinations.

50% ethanol. To extract the dye from the starch granules completely, at least 2 hr of extraction time was needed (data not shown).

The standard BSA curve gave a linear correlation between OD_{630nm} and the range of 0–250 μg of protein. The linear correlation coefficient of OD_{630nm} versus BSA in the standard curve was 0.999 at $\leq 334 \mu\text{g}$.

Extracting Starch Granule Surface Protein with SDS and 2-ME

We were unsure whether, after complete extraction of the starch granule surface protein, the starch granule could be stained with amido black 10B. It has been reported that 1% SDS solution alone does not extract the starch granule surface protein (Seguchi 1986b). However, complete extraction was done with SDS and 2-ME (Seguchi and Yamada 1989). Wheat starch granules that had been extracted with SDS and 2-ME did not stain with amido black 10B (Fig. 2B). The protein ($N \times 5.7$) levels in the extracted starch and nonextracted starch were 0.11 and 0.28%, respectively. From this data, the amount of the starch granule surface protein was calculated simply as 0.17%, in excellent agreement with 0.18% protein obtained from the dye method.

Surface Protein on Various Wheat Starch Samples Using the Dye Method

Wheat starch samples were obtained from acetic acid (pH 3.5) fractionation, air-classification, or a commercial source. Total protein levels ($N \times 5.7$) in these samples were 0.23, 0.21, and 0.27%, respectively (Table II). These samples were then tested using the dye-binding method, and the starch granule surface protein was 0.18, 0.18, and 0.16%, respectively (Table II), which indicated that 59–86% of the protein occurred on the granule surface. The dye-binding method indicated less intragranular protein in wheat starch than reported by others (Sulaiman and Morrison 1990, Skerritt and Hill 1992).

CONCLUSIONS

Wheat starch granule surface protein can be measured with a newly designed dye method using amido black 10B. Surface protein accounted for much of the protein content of three different wheat starch samples.

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