Note

Preparation of *Chalara paradoxa* Glucoamylase Modified by Fluorescein-isothiocyanate and Binding to Raw Starch[†]

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Recently Kainuma *et al.*^{1~4)} reported a kind of raw starch digesting amylase from *Chalara paradoxa*. There have been several reports on raw starch digesting amylase binding to raw starch by assaying the remaining activity or protein in the supernatant^{5,6)} In this paper, we report a

new method for studying glucoamylase binding to raw starch using fluorescein-isothiocyanate modified *Chalara paradoxa* glucoamylase.

The glucoamylase from *Chalara paradoxa* was prepared as previously reported^{3,4)} with a slight modification. Fluorescein-isothiocyanate (FITC) was coupled with the glucoamylase by the method of Hiramatsu *et al.*⁷⁾ Twenty milligrams of the glucoamylase preparation was dissolved in 1.6 ml of 0.1 M carbonate buffer (pH 8.3) and mixed with 2.5 mg of FITC ($\epsilon_{494} = 7.54 \times 10^3$, Merck) in the same buffer (0.4 ml), then incubated 30°C for 3 hr. Unreacted dye was then removed by passing through a Toyopearl 40S column and by an α -CD Sepharose 6B column.⁴⁾ This solution that contained both free glucoamylase and FITC-labelled glucoamylase was used in this study without further purification. Contamination by free dye in the sample was checked by thin layer chromatography using a silica gel 60 F₂₅₄ sheet (Merck) irrigated with 1-butanol-1-propanol-water (3:5:4); then no free dye was detected. The ratio of dye to glucoamylase was estimated to be about 0.5 by measuring the dye concentration by spectrophotometry, and the glucoamylase (M_w 78,000) con-

FIG. 1. Absorption, Excitation, and Fluorescence Spectra of FITC Modified *Chalara paradoxa* Glucoamylase.

A: absorption spectra. —, native glucoamylase; ----, free FITC, ----, FITC modified glucoamylase. The absorbance scale is arbitrary. B: excitation and fluorescence spectra of FITC modified glucoamylase. a, excitation spectrum emitted at 520 nm. b, fluorescence spectrum excited at 490 nm. C: excitation and fluorescence spectra of native and FITC modified glucoamylase. a, excitation spectra emitted at 340 nm. b, fluorescence spectra excited at 280 nm. 1, native glucoamylase; 2, FITC modified glucoamylase.

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Abbreviations: FITC, fluorescein-isothiocyanate; α -CD, alpha-cyclodextrin.

FIG. 2. Micrographs of Corn (A) and Potato (B) Starch Mixed with FITC Modified Glucoamylase. Each 2% starch digested by native glucoamylase was mixed with 200 μ l of modified glucoamylase (about 36 μ g). a, normal micrograph. b, micrograph under fluorescence microscope.

centration by Lowry's method.⁸⁾ The prepared glucoamylase had 83.4% (47.2 U/mg) of the specific activity of the original glucoamylase, and the modified glucoamylase bound to raw corn starch as well as the native enzyme; almost 80% of the activities were bound to starch granules. The binding of glucoamylase to raw starch was estimated by measuring the activity in the supernatant after incubating 2 units of glucoamylase with 100 mg of raw starch in 1 ml of acetate buffer (pH 5.0) at 40°C for 20 min. Figure 1 shows the general characteristics of absorbance and fluorescence spectra of the native glucoamylase, free FITC, and FITC-labelled glucoamylase. The labelled glucoamylase had an emission maximum at 520 nm (excited at 490 nm), and excitation maxima at the wavelengths 465 nm and 490 nm (emitted at 520 nm).

The preliminary study with native raw starches could not have revealed any difference in the binding of FITC-labelled glucoamylase because of inactivation of the glu-

coamylase or quenching of FITC fluorescence during incubation. Raw starches used in this study had been formerly digested by glucoamylase for 3 days (potato and sago) or 24 hr (corn and rice) at 40°C, in 25 mM acetate buffer (pH 5.0). The digestion percentages were 47% (potato starch), 62% (sago starch), 72% (corn starch), and 59% (rice starch). Figure 2 shows ordinary micrographs and fluorescence micrographs of potato starch and corn starch, (2% in 200 μ l) mixed with *Chalara* glucoamylase (about 5 units). These micrographs were taken with a Nikon XF-II with EFD fluorescence apparatus. As shown in Fig. 2 several granules of corn starch gave fluorescence (green color) in the whole granule, but potato starch gave fluorescence almost only on the surface. Sago starch and rice starch also had fluorescence pattern like potato starch (data not shown). As there was almost no fluorescence of the starch granules when modified glucoamylase was mixed with starch in the presence of sodium dodecyl

FIG. 3. Scanning Electron Micrograph of Starches (The bar represents 10 μ m).

Starches were digested by native glucoamylase to the digestion percentage of potato, sago, corn, and rice, 47, 62, 72 and 56%, respectively. a, potato, b, sago. c, corn. d, rice.

sulfate (SDS), we considered the fluorescence observed on starch was due to the specific binding of modified glucoamylase to starch. The scanning electron micrographs in Fig. 3 showed digestion patterns of these starches by native *Chalara paradoxa* glucoamylase. Among these starches only corn starch was digested to the center of the granule to a porous structure by digging deep cavities, but all of the other starches (potato, sago, and rice) studied here were digested probably only at the surface of the granules by digging cavities. Because of this porous structure of corn starch, under the fluorescence microscope only corn starch had fluorescence in the whole granule by binding modified glucoamylase within the holes.

In conclusion, the glucoamylase of *Chalara paradoxa* modified with FITC was bound to starches and gave fluorescence under the microscope. The pattern of fluorescence in starch granules was related to the digestion pattern by the glucoamylase.

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