Study on α-Amylase Hydrolysis of Potato Amylopectin by a Quartz Crystal Microbalance

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Potato amylopectin with phosphate groups was immobilized on a quartz crystal microbalance with dissipation monitoring (QCMD) using the attractive interaction between opposite charges, and enzymatic starch hydrolysis was monitored directly. Poly(L-lysine) (PLL) proved to be an appropriate cationic linker between the QCMD silica sensor and potato amylopectin. Increased mass and dissipation were observed when amylopectin was adsorbed onto the PLL layer and reversed when α-amylase was added. The effect of chitosan with cationic property on the hydrolysis of amylopectin was studied. Chitosan was observed to be adsorbed onto the amylopectin surface and to suppress hydrolysis by α-amylase. The formation of alternating layers of amylopectin and chitosan was monitored by QCMD. Amylopectin–chitosan trilayers increased resistance to digestion by α-amylase compared to one layer and to control without chitosan.

KEYWORDS: Amylopectin; chitosan; α-amylase hydrolysis; QCMD

INTRODUCTION

Starch is a major component in various grain crops and staple food products. Enzymatic hydrolysis of starch is an important digestive process, the rate and extent of which affect metabolic response. Slowly digested starches are generally considered to be beneficial in metabolic disorders such as diabetes (*1–3*). Resistant starch (RS) has been defined as the starch not digested and absorbed in the human small intestine (*3*). RS is considered to have benefits similar to those of dietary fiber. Changing the rate and extent of starch digestion by enzymes could induce the increase of RS and slowly digestible starch content in food. Enzymatic starch hydrolysis rate and extent are affected by such properties as the starch granule structure, crystal type, granule size, amylose/amylopectin ratio, and molecular structure and by the interaction between starch and other components (*4*). Understanding the effects of starch properties and other polymers on starch digestion thus becomes a vital issue in improving the nutritional benefit of starch and food. The extent and rate of starch hydrolysis are measured by in vitro procedures developed in attempt to mimic human starch digestion. Many such procedures use pancreatic α-amylase digestion followed by the measurement of released glucose (*5, 6*).

The quartz crystal microbalance with dissipation monitoring (QCMD) is known to provide a very sensitive mass measuring device. QCMD has been used as a tool to study real-time reaction and interaction between macromolecules (*7*). Several groups have used it to study layer-by-layer deposition of polyanions and polycations (*8–11*). The process of enzymatic hydrolysis has also been studied by QCMD in directly and quantitatively detecting negative and positive frequency shifts. Few studies, however, have dealt with its use in studying starch hydrolysis by digestive enzyme. Nishino et al. (*12*) immobilized amylopectin on the QCM electrode using ligand binding between biotin and streptavidin and detected the amylopectin hydrolysis step by glucoamylase. A technique based on alternative polyanion and polycation deposition is commonly used to form multilayers on the QCM surface. Potato starch contains a small proportion of phosphate groups linked to glucosidic chains of amylopectin (*13*). Phosphate groups covalently bind to amylopectin molecules at C-6 and C-3 of glucosyl residues (*14*). These phosphate groups make potato starch slightly anionic (*15*). In this study we investigated the enzymatic degradation of potato amylopectin immobilized on the QCM electrode by attraction between opposite charges and how the presence of other food biopolymer modifies the digestibility of starches in mixed systems. Chitosan is a natural carbohydrate polymer derived by deacetylation of chitin, a major component of the shells of crab and shrimp. Recently, the applications of chitosan have increased for the improvement of food quality (*16*). The cationic property of chitosan is appropriate for electrostatic interactions with other anionic polysaccharides. We focused on the layer formation of chitosan–starch and investigated the effects of chitosan on potato amylopectin hydrolysis by α-amylase using QCMD. Fourier transform infrared–attenuated total reflection spectroscopy (FTIR-ATR) was used to obtain information on the chemical characteristics of deposited starch–chitosan layers.

MATERIALS AND METHODS

Materials. Amylopectin from potato starch was obtained from Sigma (Sigma 10118), and a 0.6 mg/mL suspension was prepared in 10 and

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100 mM (pH 7.0) sodium acetate buffer containing 100 mM NaCl. This was then heated at 100 °C for 15 min and stored at room temperature for approximately 1 h. Poly(L-lysine) (PLL) with a mean degree of polymerization of 70 was obtained from Sigma (Sigma P6516), and a 0.6 mg/mL solution was prepared in 10 and 100 mM (pH 7.0) sodium acetate buffer containing 100 mM NaCl. α-Amylase (porcine pancreas, Sigma A6255) solution was prepared in the same buffer at the specified concentration. A 0.3 mg/mL chitosan (from crab shells, ≥85% deacetylated, Sigma C3646) solution was prepared in 100 mM (pH 4.5) acetate buffer containing 100 mM NaCl to evaluate effects on starch hydrolysis.

Quartz Crystal Microbalance. Measurements were carried out using a D300 quartz crystal microbalance with dissipation monitoring (QCMD) (Q-Sense AB, Västr Frölunda, Sweden) with a QAFC 302 axial flow measurement chamber under the same conditions as used by Krzeminski et al. (*11*). The sensing element is a disk-shaped AT-cut quartz crystal sandwiched between two gold electrodes. The sensing surface was coated with a 50 nm layer of silicon dioxide (SiO_2). An increase in mass bound to the quartz surface causes the crystal's resonant frequency to decrease. If adsorbed material is evenly distributed, rigidly attached, the frequency shift (Δf) is related to the adsorbed mass (Δm) per unit surface by the Sauerbrey equation (*17*)

$$\Delta m = -C\Delta f/n \tag{1}$$

where C = mass sensitivity constant ($C = 17.7$ ng cm^{-2} Hz^{-1} for a 5 MHz crystal), n = overtone number (in the present case $n = 1$, 3, or 5), and Δm = elastic mass change.

After the QCMD signal was stabilized in a 10 mM sodium acetate buffer (pH 7.0), a base layer was formed by a 0.6 mg/mL PLL solution in 10 mM sodium acetate buffer, followed by flushing out the redundant PLL solution with buffer. To immobilize amylopectin, a 0.6 mg/mL amylopectin suspension was allowed to flow onto the PLL layer, which was allowed to equilibrate for 20 min. The hydrolysis reaction was initiated by adding 0.5 mL of α-amylase solution. Amylopectin–chitosan layers formed on the crystal chip in 100 mM sodium acetate buffer. After amylopectin was immobilized in pH 7.0 buffer, the cell was equilibrated in pH 4.5 buffer followed by a chitosan solution flow. Multilayers of amylopectin–chitosan were prepared by repeating this with pH 7.0 buffer followed by amylopectin solution (AP), pH 7.0 buffer, pH 4.5 buffer, and chitosan solution (C) three times to give a multilayer consisting of (PLL–(AP–C)₃). After the third layer formed, redundant chitosan solution was removed with pH 4.5 and pH 7.0 buffer, followed by the hydrolysis reaction of α-amylase solution. Frequency changes were recorded at 20 °C.

FTIR-ATR Spectroscopy. For chemical characterization, layers deposited on a ZnSe crystal were studied under conditions similar to those of QCMD by FTIR spectrometer (Nicolet 860, Thermo Electron Corp., Madison, WI) fitted with a MicroCircle liquid ATR cell (SpectraTech, Warrington, U.K.). The ATR crystal was a cylindrical ZnSe prism, with 11 internal reflections, mounted in a thermostated steel jacket set at 20 °C. Infrared spectra were collected over the range of 4000–800 cm^{-1}. The PLL, amylopectin, and chitosan solution concentration was modified to detect each band peak clearly. Solutions of 1.2 mg/mL PLL and 3.0 mg/mL amylopectin in sodium acetate buffer (pH 7.0) and a 0.1 mg/mL chitosan solution in acetate buffer (pH 4.5) were prepared using D_2O instead of H_2O for FTIR analysis. α-Amylase solution was prepared in H_2O buffer (10 and 100 mM, pH7.0) at 0.01 unit/mL, because D_2O may affect the enzyme activity negatively. One milliliter of biopolymer solution was injected in the same sequence as for QCMD measurement and left for 5 min followed by 2 mL of D_2O after each injection of biopolymer. After the injection of the α-amylase solution and 5 min of standing, the cell was flushed three times with 2 mL of D_2O buffer (pH 7.0) and the appreciable absorption band of water at 1643 cm^{-1} (O–H bending) was confirmed to disappear, because the amide I band is strongly affected by this absorption band.

RESULTS AND DISCUSSION

Potato Amylopectin Hydrolysis by α-Amylase in QCMD Measurement. Figure 1 shows the frequency and dissipation changes in the hydrolysis of potato amylopectin immobilized
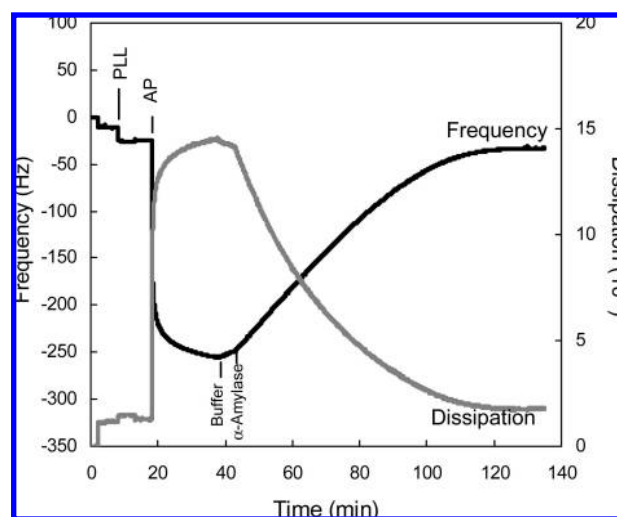

Figure 1. QCMD frequency (black line) and dissipation (gray line) shifts during the deposition of PLL and potato amylopectin and hydrolysis by α-amylase (0.005 unit/ml) in 10 mM sodium acetate buffer.

on a QCMD over time. For clarity, only QCMD data for the third harmonic (15 MHz) is reported here. The frequency rapidly decreased when PLL solution was added, indicating that PLL bound to the silica surface of QCMD plate. The hydrated mass of bound PLL calculated by the Sauerbrey equation (eq 1) was 71.6 ± 4.6 ng cm^{-2}. The silica QCMD plate has the anionic surface, which adsorbs the cationic but not the anionic starch (*18*). Potato amylopectin is anionic, containing a small amount of phosphate groups (*13*). Multiply charged PLL has been selected for adsorbed polycation due to its electrostatic attraction and the interaction with other polymers in many studies (*11, 19–21*). In this study, PLL was expected to immobilize potato amylopectin as an intermediary on the silica QCMD plate. Rinsing with buffer after the addition of PLL slightly increases the frequency and decreases in dissipation, which suggests that a small amount of PLL weakly immobilized on the silica surface was washed away. When a potato amylopectin solution was added, the resonant frequency swiftly decreased due to the adsorption of amylopectin on the PLL base layer, indicating that the interaction of opposite charges between PLL and potato amylopectin immobilized the amylopectin on the QCMD plate. When waxy rice starch was allowed to flow onto the PLL layer instead of potato amylopectin, no frequency change was observed, indicating that waxy rice starch has too few phosphate groups to generate sufficient ionic charge. The frequency shift when potato amylopectin was added was extremely large. The QCMD technique detects changes in hydrated mass adsorbed on the electrode surface. Potato amylopectin has a much higher molecular weight than PLL and a high swelling capacity, which causes a great increment of the hydrated mass (1308.4 ± 42.7 ng cm^{-2}) followed by increased dissipation. The dissipation (D) of the crystal's oscillation is associated with the measuring of the softness or viscoelasticity of deposited layers. D is defined as

$$D = E_{lost}/2\pi E_{stored}$$

where E_{lost} is energy lost during one oscillation cycle and E_{stored} is total energy stored in the oscillator. The large dissipation indicates that potato amylopectin formed a highly viscoelastic layer on PLL, rather than a rigid layer.

Using QCMD enabled us to directly monitor potato amylopectin as α-amylase enzymatically degraded it. As shown in **Figure 1**, potato amylopectin immediately undergoes hydrolysis when α-amylase solution is added. The enzyme binding to

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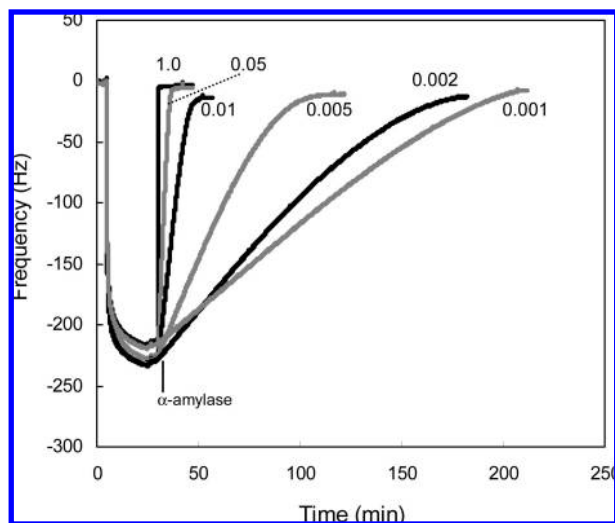*J. Agric. Food Chem.*, Vol. 56, No. 3, 2008   **1093**

Figure 2. Time courses of frequency changes during the hydrolysis of potato amylopectin immobilized on QCMD at different concentrations of α-amylase (0.001, 0.002, 0.005, 0.01, 0.05, 1.0 unit/mL).

Figure 3. Relationships between initial hydrolysis rates of amylopectin (v_0) calculated from curve obtained by QCMD and concentrations of α-amylase.

Figure 4. QCMD frequency (black line) and dissipation (gray line) shifts during the deposition of potato amylopectin−chitosan layers and hydrolysis by α-amylase (0.01 unit/mL) in 100 mM buffer.

amylopectin was not observed as an increase in mass. If α-amylase irreversibly binds to the amylopectin layer surface, the binding of enzyme molecules could be directly observed by QCMD. Monitoring the process of amyloglucosidase binding amylopectin by QCM has been reported (*12*), and we observed the same phenomenon in adding amyloglucosidase to potato amylopectin immobilized on the PLL layer. It is considered that the active site of porcine pancreatic α-amylase has five subsites involved in the binding of glucose units (*22*). The adsorption of *Bacillus subtilis* α-amylase specifically onto spherulitic starch particles is a step essential for hydrolysis (*23*). The enzyme–substrate complex must form for the initial enzyme action on the substrate. Amyloglucosidase successively catalyzes the hydrolysis of terminal 1,4-linked α-D-glucose residues from the nonreducing ends of starch with β-D-glucose release, whereas α-amylase catalyzes the hydrolysis of internal α-(1,4) glucosidic bonds in starch and cannot cleave the α-(1,6) bond. The products of α-amylase are dextrin and an oligomer mixture, suggesting that α-amylase hydrolysis may start immediately after enzyme is added and release a mass of dextrin or oligomer much greater for hydrolysis than the increased mass for the formation of the enzyme–substrate complex.

Figure 2 shows time courses of frequency changes observed for α-amylase potato amylopectin hydrolysis at different enzyme concentrations. The frequency gradually increased due to the hydrolysis, reaching an approximately constant value independent of enzyme concentration and removing almost all of the immobilized amylopectin after hydrolysis. The rate of amylopectin hydrolysis by α-amylase increased with the enzyme concentration. The hydrated masses of immobilized and released potato amylopectin calculated by the Sauerbrey equation (eq 1) were 1308.4 ± 42.7 and 1263.0 ± 25.4 ng cm^{-2}, respectively. Mass calculation showed $96.6 \pm 1.9\%$ of amylopectin released by α-amylase hydrolysis. Because the time course of frequency changes in QCMD was linear with time for the initial part, the initial hydrolysis rates (v_0) was obtained from curve fittings of the initial frequency increase after α-amylase was added. **Figure 3** shows the plot of the initial hydrolysis rates of potato amylopectin against the concentration of added α-amylase solution. The initial amylopectin hydrolysis rate increased linearly with α-amylase concentration in double-logarithmic approximation ($R^2 = 0.986$).
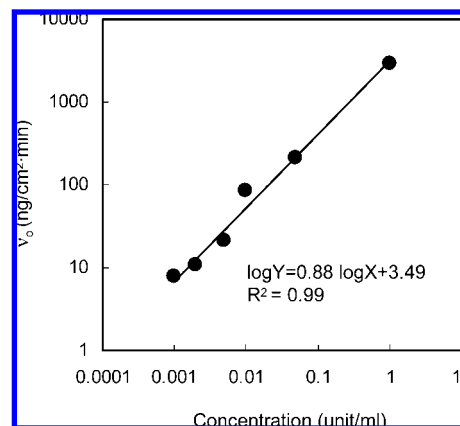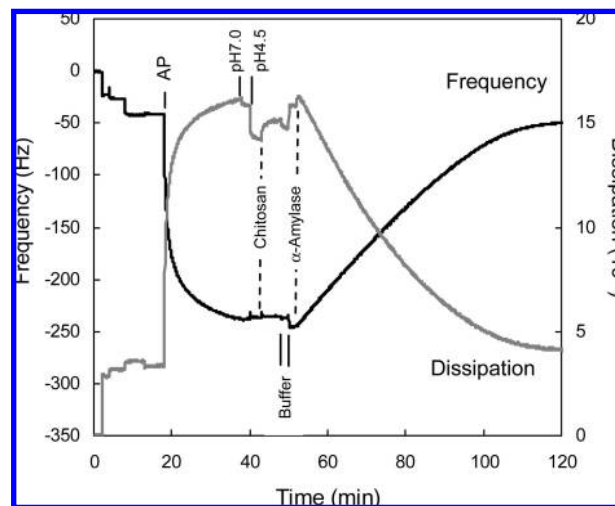
The interaction between potato amylopectin and chitosan was monitored directly by QCMD (**Figure 4**). When the acidic buffer (pH 4.5) was used for rinsing after the addition of amylopectin, a much greater reduction in dissipation was found compared with pH 7.0 buffer. This suggests that the change in pH strongly influenced the viscoelastic properties of amylopectin layer. When 0.3 mg/mL chitosan solution in 100 mM (pH 4.5) acetate buffer was added to immobilized amylopectin, a slight reduction in the hydrate mass (-7.6 ± 0.3 ng cm^{-2}) and an increase in dissipation [$(0.95 \pm 0.01) \times 10^{-6}$] were observed, indicating that the amylopectin−chitosan layer was built up with the shrinkage due to chitosan binding. Chitosan is the deacetylated product of chitin, which is the second most abundant naturally occurring biopolymer. A cationic heteropolysaccharide, it consists mainly of β-(1,4)-2-deoxy-2-amino-D-glucopyranose units and partially of β-(1,4)-2-deoxy-2-acetamido-D-glucopyranose. Studies have been done on the film formation of chitosan and other polymers (*24–28*). The cationic property of chitosan has potential for electrostatic interactions with other anionic polysaccharides such as potato amylopectin. Combining chitosan and starch reportedly formed a film (*29–31*). Adding chitosan increased dissipation, suggesting that cationic chitosan binds negatively charged potato amylopectin with phosphate groups by opposite charges attracting. In the process of washing with pH 4.5 buffer followed by pH 7.0 buffer, negative shifts in
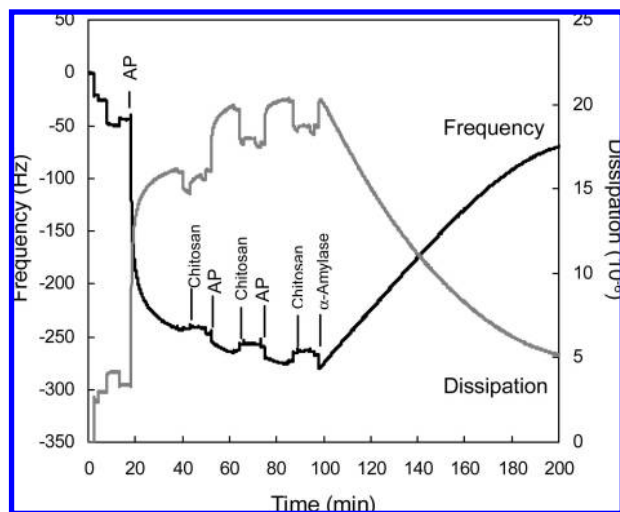
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Figure 5. QCMD frequency (black line) and dissipation (gray line) shifts during the deposition of potato amylopectin–chitosan trilayers and hydrolysis by α-amylase (0.01 unit/mL) in 100 mM buffer.

Figure 6. Effect of chitosan deposition on the hydrolysis rate of potato amylopectin immobilized on QCMD: (a) amylopectin (control); (b) amylopectin–chitosan layers; (c) trilayers of amylopectin–chitosan.

Figure 7. Deposition of PLL (black line) and potato amylopectin (gray line) in 10 mM D_2O buffer and 5 min hydrolysis of potato amylopectin by α-amylase (dashed line) followed by FTIR-ATR.

frequency, meaning an increase in the hydrated mass, and increased dissipation were observed in the two stages. Results indicate that potato amylopectin and chitosan layers possess high hydrate capacity and viscoelasticity after washing with buffer. We studied the formation of amylopectin–chitosan trilayers on the QCMD electrode (**Figure 5**). When the second and third amylopectin layers were added to the chitosan layer, a negative frequency change was observed, indicating that potato amylopectin was adsorbed onto the chitosan surface by opposite charges attracting and forming alternating layers of amylopectin and chitosan. The deposition of amylopectin gradually decreased, showing a distinct difference in amount between the first and second depositions. Amylopectin was initially deposited by opposite charges attracting between PLL and amylopectin, and the second and third depositions were made by the attraction between chitosan and amylopectin. The graduated reduction of amylopectin deposition may be due to decreasing charge availability. Dissipation indicated that viscoelasticity increased as the amylopectin–chitosan layer was deposited. We compared the initial α-amylase hydrolysis rates (v_0) from curve fittings between amylopectin–chitosan layers and control without chitosan (**Figure 6**). The initial amylopectin hydrolysis rate of control was 41.5 ng cm^{-2} min^{-1} ($R^2 = 0.995$), that of amylopectin–chitosan layer, 21.7 ng cm^{-2} min^{-1} ($R^2 = 0.995$), and that of trilayers, 13.0 ng cm^{-2} min^{-1} ($R^2 = 0.998$). The deposition of chitosan on potato amylopectin suppressed the enzymatic hydrolysis of amylopectin, suggesting that the chitosan coating modulated the access of enzyme to starch granules, possibly resulting in a barrier material for starch digestion.

FTIR-ATR Spectroscopy. Figure 7 shows FTIR spectra for the deposition of PLL, amylopectin, and α-amylase hydrolysis. The deposition of each layer showed characteristic spectral changes. The amide I band of PLL in a random coil conformation has an absorbance of 1643–1648 cm^{-1} (*32*). The initial deposition of PLL on the ZnSe crystal surface had absorbance characteristic of these groups. After potato amylopectin was added, the typical band of C–O stretching for glucose appeared at 1032 and 1159 cm^{-1} (*33–35*). The results supported QCMD experiment observations, which involve the formation of PLL and amylopectin layers by opposite charges attracting. We confirmed that the potato amylopectin solution was not directly adsorbed onto the ZnSe crystal surface. The addition of
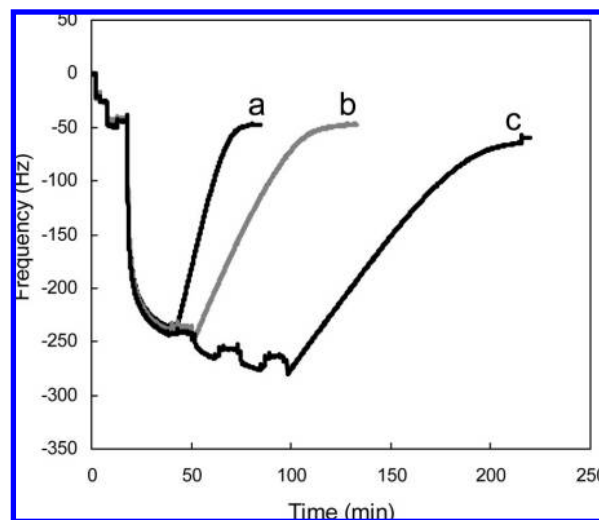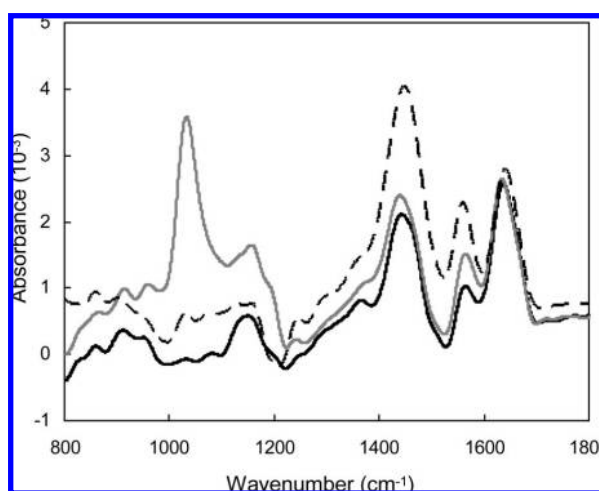
α-amylase (0.01 unit/mL) decreased the absorbance at 1032 and 1159 cm^{-1} due to amylopectin hydrolysis. This supports absorbance at 1032 and 1159 cm^{-1} as proceeding from amylopectin. Changes in frequency for the deposition of chitosan onto amylopectin were studied for comparison to QCMD experiments. When chitosan was added, amide peak absorbance decreased at 1643–1648 cm^{-1}, similar to a previous finding on the deposition of PLL and pectin (*11*), suggesting some stripping of PLL from layers with chitosan under the condition of the FTIR experiment. The C=O band for chitosan was reported to appear at 1620 and 1660 cm^{-1} (*35*). However, this band was indistinguishable from the amide band of PLL in this study. Trilayers of amylopectin–chitosan formed on the ZnSe crystal surface under the same conditions as in the QCMD experiment. The deposition of amylopectin was detected in three stages by increased absorbance at 1032 cm^{-1} (**Figure 8**). After hydrolysis by α-amylase for 5 min, residual amylopectin resistant to digestion was observed at 1032 cm^{-1}. When α-amylase was added to amylopectin without a chitosan coating, this peak almost disappeared after 5 min (**Figure 7**), indicating that the chitosan coating is effective for modifying the rate of starch digestion, consistent with QCMD experiment results. The
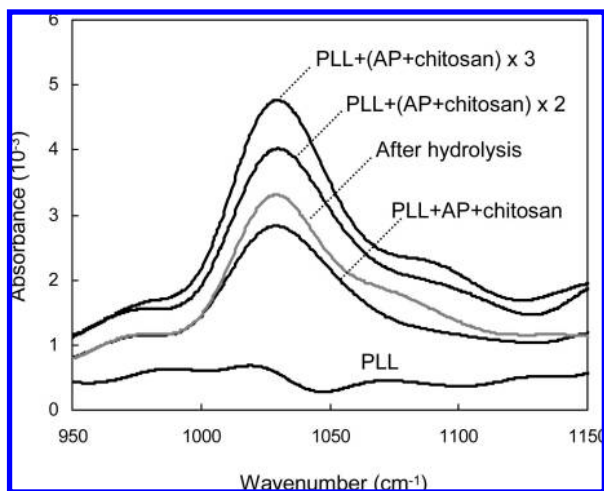
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Figure 8. Changes of FTIR spectra for the successive deposition of potato amylopectin and chitosan (black lines) and spectra after 5 min hydrolysis by α-amylase (gray line).

interaction between potato amylopectin and chitosan was observed by the real-time measurement using QCMD to suppress the rate of α-amylase hydrolysis; however, the effects of the other polymers on starch hydrolysis should be investigated further.

Conclusions. The hydrolysis of potato amylopectin by α-amylase was directly monitored by QCMD. PLL proved to be an appropriate linker for immobilizing potato amylopectin on the silica surface of the QCMD plate by opposite charge attraction. Adding potato amylopectin resulted in a large increase in hydrated mass and dissipation. Adding α-amylase immediately reduced the hydrated mass as amylopectin was hydrolyzed. The initial rate of amylopectin hydrolysis was calculated from curves obtained in QCMD experiments. The impact of chitosan on amylopectin hydrolysis was observed by QCMD. Chitosan with cationic properties was adsorbed onto the anionic surface of potato amylopectin. The adsorbed chitosan suppressed the rate of amylopectin hydrolysis. Repeatedly adding amylopectin and chitosan formed alternating trilayers of amylopectin−chitosan by opposite charge attraction, and the deposition of these layers was also observed on a ZeSe crystal by FTIR-ATR. Our results suggest that chitosan potentially provides a modified surface layer of potato amylopectin, modulating the access of enzyme.

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