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Effects of protein on crosslinking of normal maize, waxy maize, and potato starches

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Abstract

Channels of maize starch granules are lined with proteins and phospholipids. Therefore, when they are treated with reagents that react at or near the surfaces of channels, three types of crosslinks could be produced: protein–protein, protein–starch, starch–starch. To determine which of these may be occurring and the effect(s) of channel proteins (and their removal) on crosslinking, normal and waxy maize starches were treated with a proteinase (thermolysin, which is known to remove protein from channels) before and after crosslinking, and the properties of the products were compared to those of a control (crosslinking without proteinase treatment). After establishing that treatment of starch with thermolysin alone had no effect on the RVA trace, three reaction sequences were used: crosslinking alone (CL), proteinase treatment before crosslinking (Enz-CL), proteinase treatment after crosslinking (CL-Enz). Two crosslinking reagents were used: phosphoryl chloride ($POCl_3$), which is known to react at or near channel surfaces; STMP, which is believed to react throughout the granule matrix. Three concentrations of $POCl_3$ (based on the weight of starch) were used. For both normal maize starch (NMS) and waxy maize starch (WMS) reacted with $POCl_3$, the trends were generally the same, with apparent relative degrees of crosslinking indicated to be CL-Enz = CL > Enz-CL, but the effects were greater with NMS and there were differences when different concentrations of reagent were used. The basic trends were the same when potato starch was used in the same experiments. Crosslinking with STMP was done both in the presence and the absence of sodium sulfate (SS). Both with and without SS and with both NMS and WMS, the order of indicated crosslinking was generally the same as found after reaction with $POCl_3$, with the indicated swelling inhibition being greater when SS was present in the reaction mixture. Examination of the maize starches with a protein stain indicated that channel protein was removed by treatment with thermolysin when the proteinase treatment occurred before crosslinking with either $POCl_3$ or STMP, but only incompletely or not at all if the treatment with the proteinase occurred after crosslinking. Because the crosslinking reactions were less effective when the protein was removed, the results are tentatively interpreted as indicating that they involved protein molecules, although there may not be a direct relationship.

Keywords: Crosslinked starch; Normal corn starch; Potato starch; Waxy corn starch; Starch granule protein

1. Introduction

Previously, starch protein was categorized as surface and granule-bound (Gillian, Sargeant, & Schofield, 1981). In isolated maize starch, the external surface protein is zein, which can be removed by treatment with thermolysin) (Han, Benmoussa, Gray, BeMiller, & Hamaker, 2005; Tester, Yousuf, Kettlitz, & Röper, 2007). Among granule-bound proteins are the waxy protein (granule-bound starch synthase, GBSS) (Nakamura, Yamamori, Hirano, & Hidaka, 1993), starch synthase (SSI), and starch branching enzyme IIb (SBEIIb) (Mu-Forster et al., 1996). They are extracted only when the starch granules are considerably swollen (Sano, 1984). Like the external surface protein, the protein components of the lining of granule channels of normal and waxy maize starch (Fannon, Gray, Guna-

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wan, Huber, & BeMiller, 2003, 2004; Han, Gray, Huber, & BeMiller, 2006) can be removed by treatment with thermolysin (Han et al., 2005).

A widely used type of chemical derivatization used to make modified food starches is that which introduces distarch phosphate crosslinking. Phosphoryl chloride ($POC1_3$) is the most widely used reagent for crosslinking. Its reaction with starch is very rapid, resulting in reactions at or near the external and channel surfaces of corn starch granules (Gray & BeMiller, 2004; Huber & BeMiller, 2001). Because it reacts at or near the surfaces of channels, it could produce three possible types of crosslinks: protein–protein, protein–starch, or starch–starch. Reactions with sodium trimetaphosphate (STMP) and epichlorohydrin (EPI), two additional crosslinking reagents, are much slower and are, therefore, believed to occur more evenly throughout granules. Hirsch and Kokini (2002) compared reactions of waxy maize starch with $POC1_3$, STMP, and EPI and reported that $POC1_3$-generated crosslinks appeared to be much more effective in preventing granule swelling than were those resulting from reaction with STMP or EPI.

Reagents contact granule surfaces, including channel surfaces, before penetrating into the matrix; so it was hypothesized that removing the protein lining granule channels of maize starch could affect chemical modification. The objective of this research was to determine the effect of channel and surface proteins on modification of normal and waxy maize starches. For crosslinking, phosphoryl chloride ($POCl_3$) and STMP were used. Pasting characteristics were used to determine the degree of reaction. Potato starch, which is devoid of surface pores (Fannon, Hauber, & BeMiller, 1992) and channels, was reacted in the same ways for comparison.

2. Materials and methods

2.1. Materials

Commercial normal and waxy maize (Tate & Lyle North America, Decatur, IL) and potato starches (Penford Food Ingredients, Englewood, CO) were used. A CBQA protein quantitation kit containing 3-(4-carboxybenzoyl)quinoline-2-carboxyaldehyde was purchased from Molecular Probes (Eugene, OR). Thermolysin was obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO).

2.2. Crosslinking of starch with phosphoryl chloride ($POCl_3$)

Starch (20 g, db) was reacted with three levels of $POCl_3$ (0.05%, 0.075%, and 0.1% [0.53, 0.79, and 1.06 mmol/glucosyl unit] based on the dry weight of starch) at pH 11.2 for 1 h at 25 °C. Total slurry volume was 40 mL. Reaction pH (11.2) was maintained by addition of 1 M NaOH using a pH-stat autotitrator (Copenhagen, Denmark). After reaction, the starch slurry was neutralized and recovered by vacuum filtration, washed with water and absolute ethanol, and air-dried.

2.3. Crosslinking of starch with sodium trimetaphosphate (STMP)

The method described by Lim and Seib (1993) was used.

2.4. Incubation of starch with thermolysin

Treatment of starch granules with thermolysin (Sigma Chemical Co., St. Louis, MO) was conducted as described by Mu-Forster and Wasserman (1998).

2.5. Confocal laser scanning microscopy (CLSM)

The procedures described by Han et al. (2005) were used.

2.6. RVA analysis

The pasting characteristics of the starches were determined with a Rapid Visco-Analyser (Model 4, Newport Scientific, Warriewood, Australia) using standard profile 1. A 13-min analysis was used: equilibration to 50 °C for 1 min, heating to 95 °C in 222 s, holding at 95 °C for 150 s, cooling to 50 °C in 228 s, and holding at 50 °C for 120 s. Unmodified and treated starches (2.1 g, db) and 27.9 g of distilled water were combined and stirred in the aluminum sample container for 20–30 s before inserting the container into the instrument. Analyses were done in triplicate.

3. Results and discussion

Starch samples treated with thermolysin (2 mg/g starch) and without treatment (1 g starch only) were analyzed by the Somogyi–Nelson method (Wood, 1994) to check the extent of starch hydrolysis due to contamination of the thermolysin preparation with amylase. The amount of reducing sugar (determined using D-glucose as a reference) was 111 μg/g starch and 22 μg/g starch, respectively, for the starches with and without thermolysin treatment. Although this degree of hydrolysis was thought to be insignificant, to minimize any effect of depolymerization, we determined the approximate minimal concentration of enzyme required (using CLSM); 1 mg/g starch was the amount determined and used thereafter.

3.1. Confocal laser scanning microscopy (CLSM)

Thermolysin is a metaloproteinase that catalyzes hydrolysis of proteins at both protein–membrane and protein–carbohydrate interfaces (Sigma–Aldrich Chemical Co., 1999). Using this enzyme, the main surface protein of starch granules, zein, and most of the channel protein could be removed in a 30-min treatment (Han et al., 2005). Fig. 1 contains CLSM pictures of (1) native and

(2) thermolysin-treated waxy maize starch. As Han et al. (2005) reported, channel and surface proteins were almost completely removed from unmodified waxy maize starch by treatment with thermolysin. CLSM pictures of granules crosslinked with STMP (Fig. 1 – 3) or with $POCl_3$ (Fig. 1 – 5) were almost the same as those of native waxy maize starch, indicating that protein was still present. Crosslinked, then proteinase-treated starch (Fig. 1 – 4, 1 – 6) was different from enzyme-treated, unmodified waxy maize starch (Fig. 1 – 2), as some channel and surface proteins remained (white arrows) in the crosslinked starches. It appears that crosslinking reagents (STMP and $POCl_3$) form bridges between starch and protein or protein and protein (inter- or intramolecularly) so that the proteins are not completely removed by treatment with thermolysin. Differences in the CSLM micrographs of granules crosslinked with $POCl_3$ and STMP were not obvious.

3.2. Pasting properties of $POCl_3$ -crosslinked starch before and after enzyme treatment

The pasting profiles of unmodified normal and waxy maize starches that had been treated with thermolysin showed no significant changes in either pasting temperature or peak viscosity (Enz [Figs. 2, 3, 5, 6]) when compared with those of native starch (Unmodified [Figs. 2, 3, 5, 6]), although CLSM revealed that most of the surface and channel protein had been removed (Fig. 1). Derycke

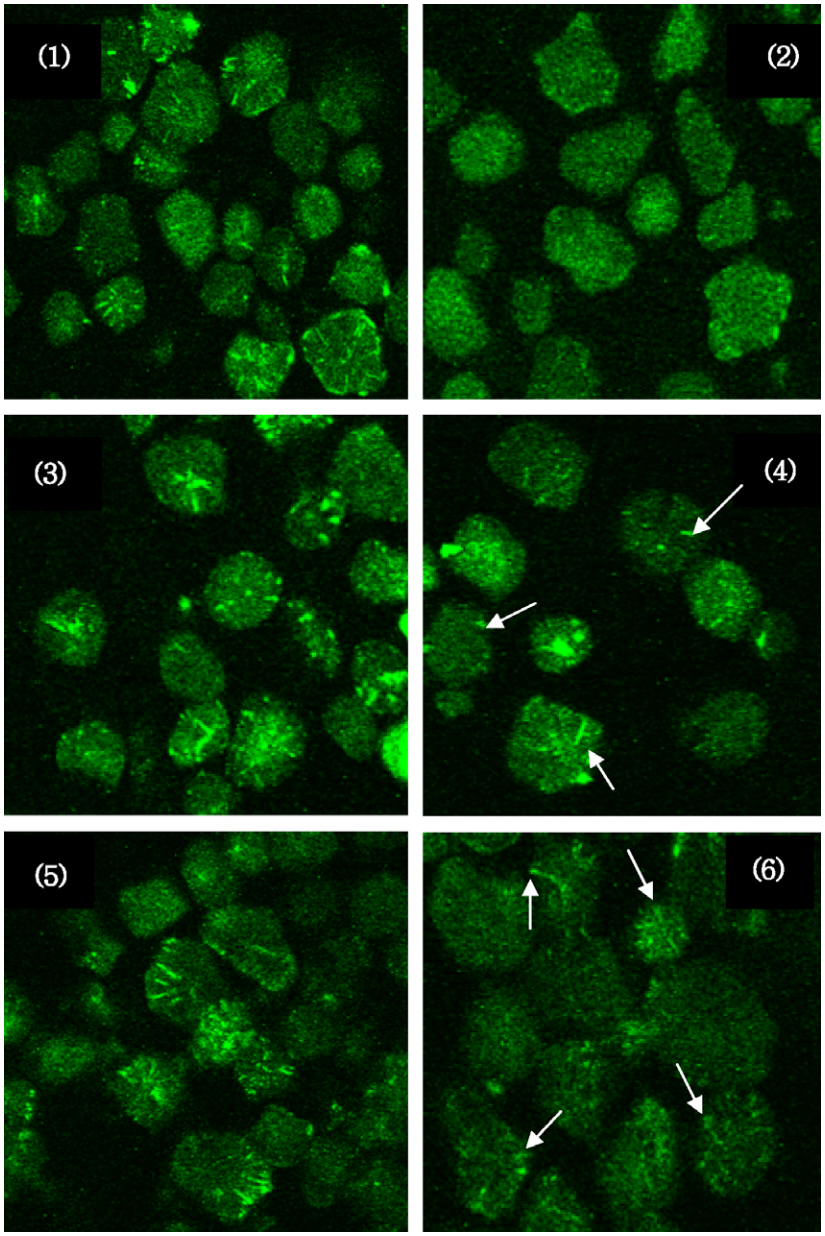

Fig. 1. CLSM micrographs of CBQCA-treated starches showing the location of protein: (1) native waxy maize starch, (2) thermolysin-treated waxy maize starch, (3) waxy maize starch crosslinked (with STMP, 0.05%), (4) crosslinked (STMP), then thermolysin-treated, waxy maize starch, (5) crosslinked waxy maize starch (0.05% $POCl_3$), and (6) crosslinked ($POCl_3$), then thermolysin-treated, waxy maize starch.

et al. (2005) reported that paste viscosity decreased after treatment of rice and rice flour with tryspin and attributed the decrease to the breaking of disulfide bonds. Lim, Lee, Shin, and Lim (1999) reported that removal of protein using 0.1% and 0.25% NaOH and 1.2% dodecylbenzene

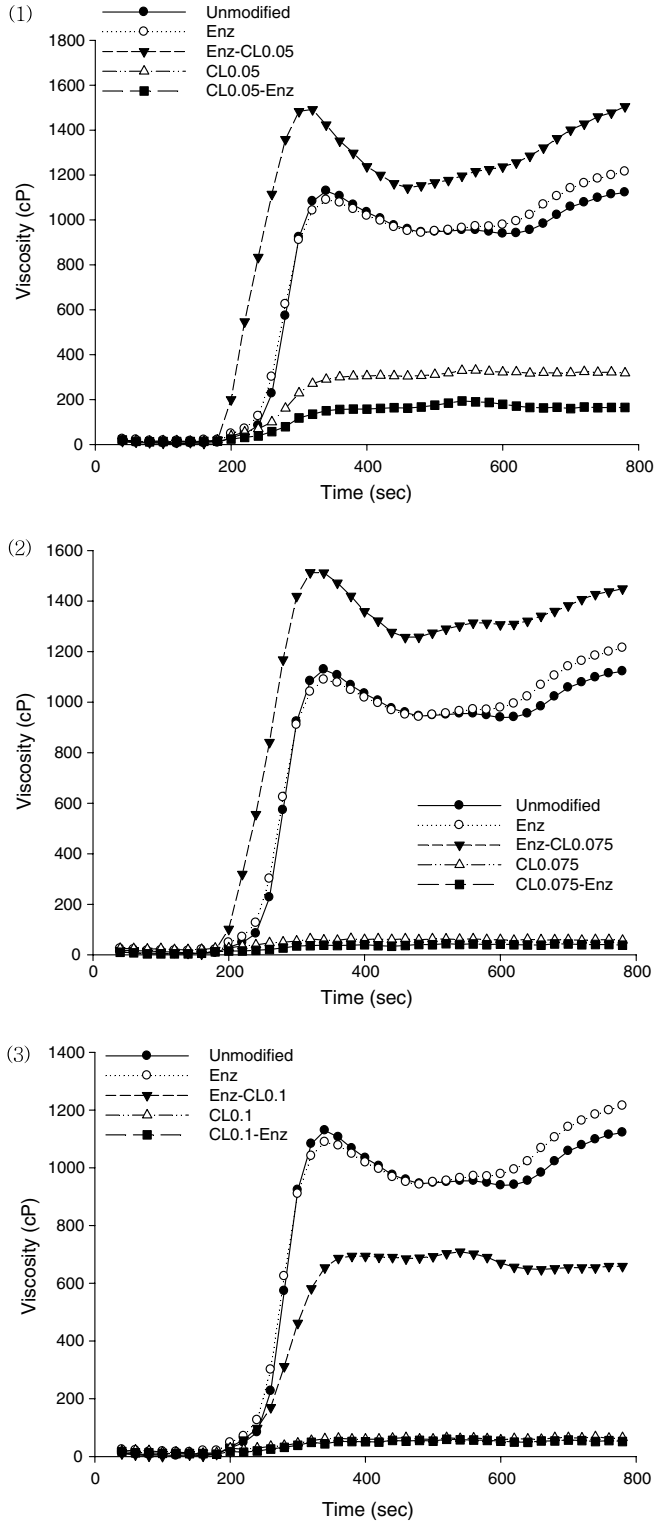

Fig. 2. RVA profiles of crosslinked normal maize starch [(1) 0.05%, (2) 0.075%, (3) 0.1% $POCl_3$] before and after treatment with thermolysin.

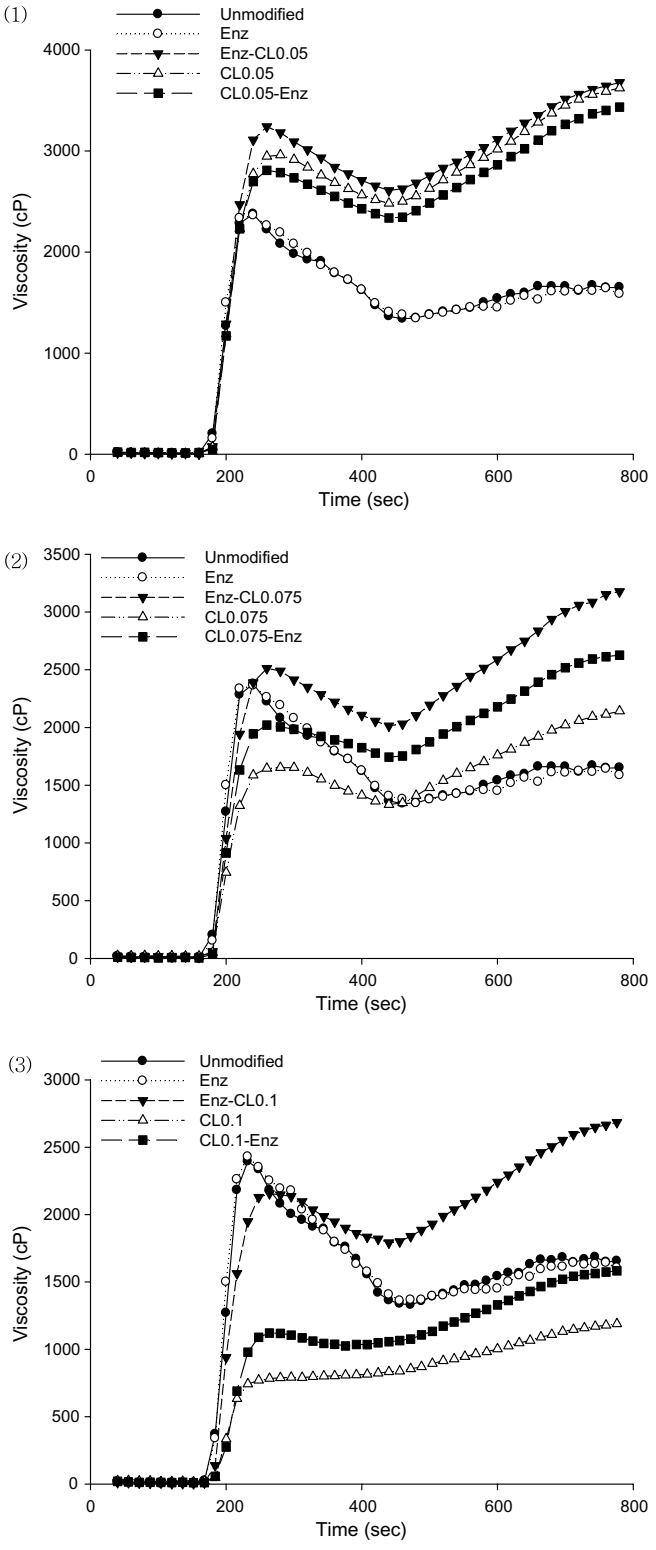

Fig. 3. RVA profiles of crosslinked waxy maize starch [(1) 0.05%, (2) 0.075%, (3) 0.1% $POCl_3$] before and after treatment with thermolysin.

sulfonate or 1.2% sodium lauryl sulfate containing 0.12% sodium sulfite affected the pasting properties of rice starch, an indication that the pasting and paste characteristics of rice starch are affected by protein content. The peak viscos-

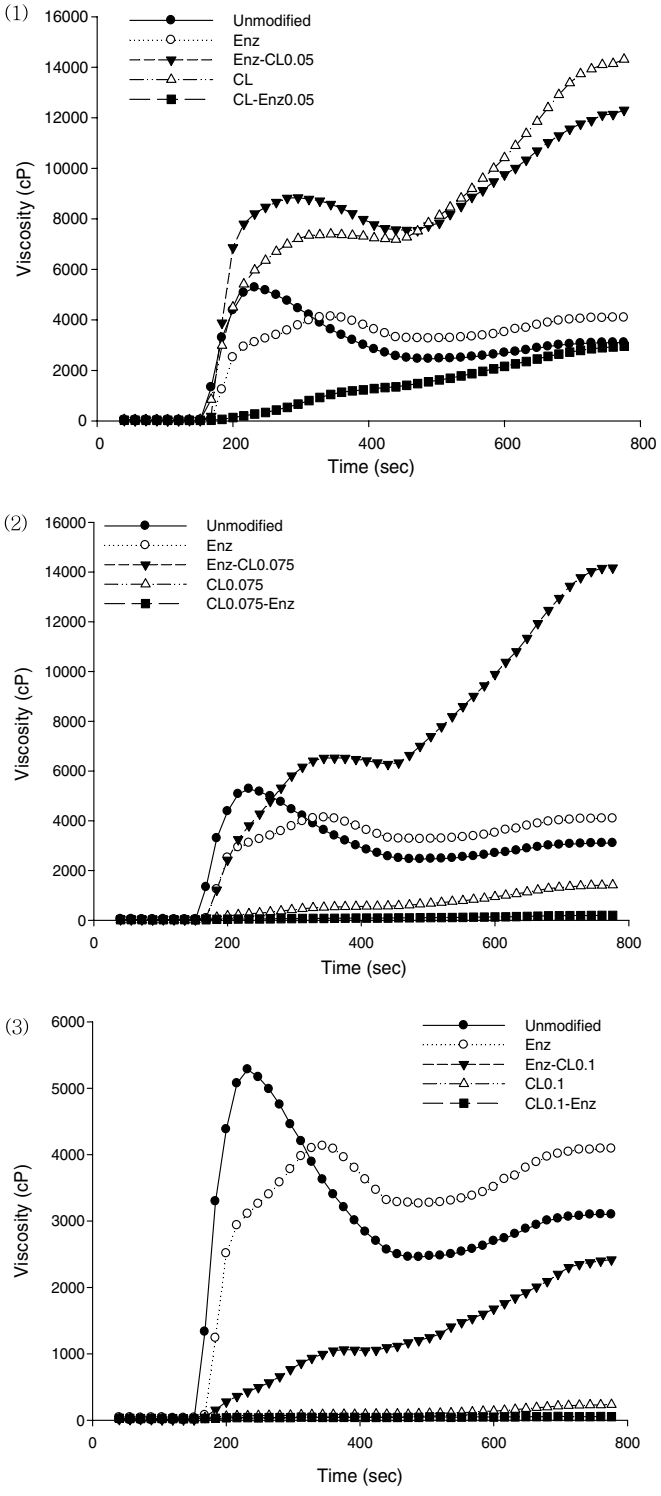

Fig. 4. RVA profiles of crosslinked potato starch [(1) 0.05%, (2) 0.075%, (3) 0.1% $POCl_3$] before and after treatment with thermolysin.

ity increased and the pasting temperature decreased upon removal of protein using these procedures. These differing reports suggest that the different protein removing methods, especially those involving use of high pH solutions or detergents, probably affect the natures of the starch granules themselves, in addition to removing protein.

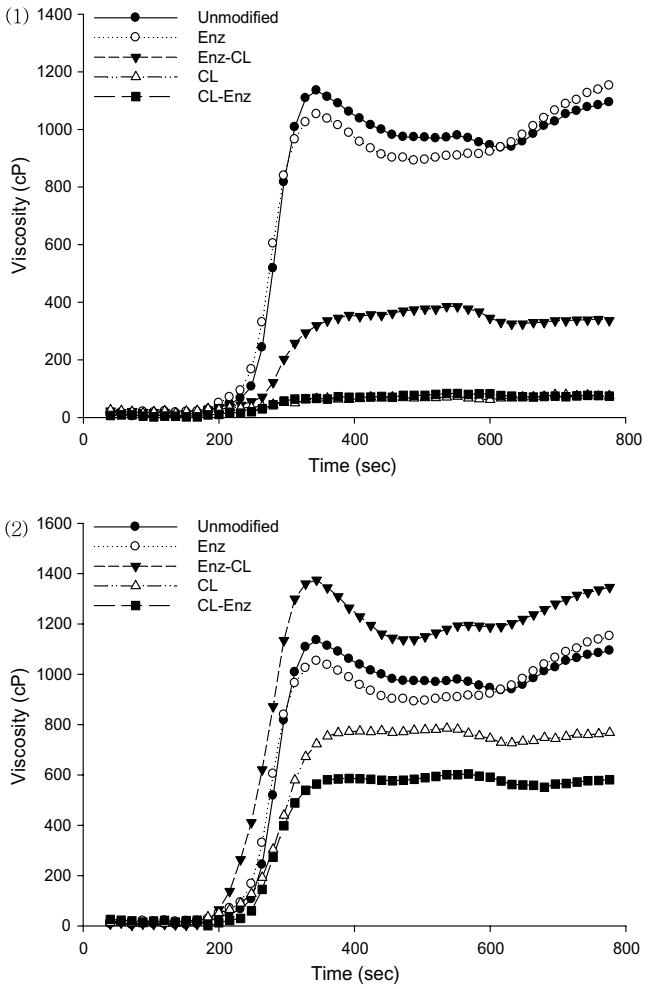

Fig. 5. RVA profiles of crosslinked (STMP) normal maize starch [(1) with SS, (2) without SS)] before and after treatment with thermolysin.

For normal maize starch, treatment with the proteinase before all three levels of crosslinking with $POCl_3$ (Enz-CL0.05 [Fig. 2 – 1], Enz-CL0.075 [Fig. 2 – 2], and Enz-CL0.1 [Fig. 2 – 3]) produced starches that were much less inhibited than those produced by crosslinking alone (CL) (CL0.05 [Fig. 2 – 1], CL0.075 [Fig. 2 – 2], and CL0.1 [Fig. 2 – 3]), for their RVA profiles showed much higher peak viscosities and greater breakdown as compared to the starches that were crosslinked without prior treatment with thermolysin. It is clear that protein removal affects the apparent extent of crosslinking, i.e., the reaction appears to be less efficient when the protein is removed. Also, the pasting temperature was reduced when the protein was removed before crosslinking when the two lowest amounts of crosslinking reagent were used (Enz-CL0.05 [Fig. 2 – 1] and Enz-CL0.075 [Fig. 2 – 2]), but this effect was not found when the higher amount of $POCl_3$ (0.1%) was used (Enz-CL0.1 [Fig. 2 – 3]). This effect of reducing the pasting temperature was not found when the starch was only treated with the proteinase (Enz [Fig. 2]). It might be that the granules were more sensitive to the alkaline conditions used in the crosslinking reaction (pH 11.2) after

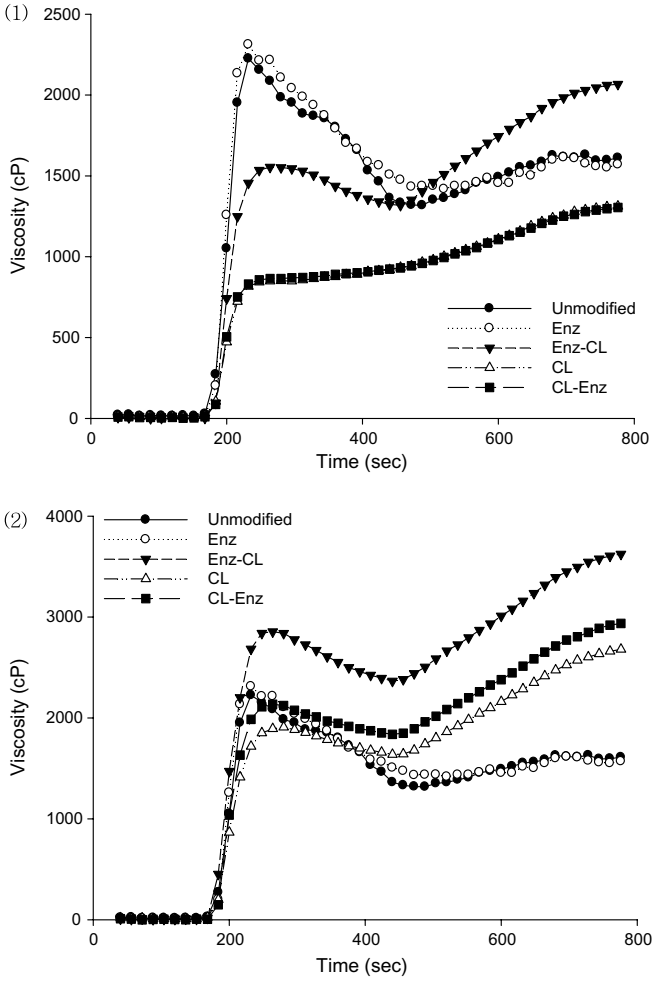

Fig. 6. RVA profiles of crosslinked (STMP) waxy maize starch [(1) with SS, (2) without SS)] before and after treatment with thermolysin.

protein removal and that the granules were changed by these conditions.

When treatment with thermolysin was done after crosslinking, no differences were found in the apparent degrees of crosslinking as compared to crosslinking without subsequent treatment with the proteinase when the higher amounts of $POCl_3$ (0.075% and 0.10%) were used (CL0.075-Enz [Fig. 2 – 2] and CL0.1-Enz [Fig. 2 – 3] vs. CL0.075 [Fig. 2 – 2] and CL0.1 [Fig. 2 – 3]), i.e., enzyme-catalyzed cleavage, if any, of the protein in a $POCl_3$-crosslinked starch still resulted in an inhibited starch. A small difference was found when the lowest amount of $POCl_3$ (0.05%) was used (CL0.05-Enz [Fig. 2 – 1] vs. CL0.05 [Fig. 2 – 1]).

Differences in the RVA profiles between Enz-CL, CL, and CL-Enz were very small in the case of lightly crosslinked waxy maize starch (Fig. 3). As the amount of $POCl_3$ used was increased from 0.05% to 0.075% and then to 0.1%, differences increased, and the peak and final viscosities were in the following order: Enz-CL > CL-Enz > CL. With crosslinked normal and waxy maize starch products, the paste and final viscosities of Enz-CL were always greater than that of CL or CL-Enz. There were, however, three differences between normal corn and waxy maize starches: (1) there was basically no difference in pasting temperature among the products from waxy maize starch treated with thermolysin before crosslinking (in contrast to normal corn starch); (2) with the exception of the use of the least amount of crosslinking reagent, CL-Enz waxy maize starch showed greater peak and final viscosities than did CL, while for normal maize starch, CL-Enz was almost the same as CL; and (3) while CL and CL-Enz produced highly inhibited starches with normal maize starch, only moderately inhibited starches were produced by the same treatments on waxy maize starch.

Enzyme treatment alone of potato starch, used for comparison because it does not have pores (Fannon et al., 1992) or channels, produced a change in the pasting curve, viz., a slightly higher pasting temperature, a lower peak viscosity, and reduced breakdown (Enz [Fig. 4]) as compared to untreated potato starch (Unmodified [Fig. 4]). Except when the lowest concentration of $POCl_3$ (0.05%) was used, Enz-CL produced the same effects in potato starch (Enz-CL0.075 [Fig. 4 – 2] and Enz-CL0.1 [Fig. 4 – 3]) as in the maize starches, i.e., crosslinking following proteinase treatment (Enz-CL) gave products that produced pastes with higher peak and final viscosities than did CL or CL-Enz. Among the crosslinked potato starch products, CL-Enz was the most inhibited, as was true for normal maize starch. In general, removing protein before crosslinking had much less of an effect on the crosslinking reaction in potato starch as compared to the maize starches, especially when 0.05% $POCl_3$ was used (Enz-CL0.05 [Fig. 4 – 1] and CL0.05 [Fig. 4 – 1]).

3.3. Pasting properties of STMP-crosslinked starch before and after enzyme treatment

Evidence that reaction with STMP produces crosslinks somewhat evenly distributed throughout the granule matrix rather than being concentrated near surfaces was given by the fact that, while phosphate ester groups that the result from reaction with $POCl_3$ can be located at the external and, especially, the channel surfaces of granules using the technique of Gray and BeMiller (2004), presumably due to the clustering of Ag atoms and thus phosphate ester groups there, they could not be located in granules modified with STMP, even though greater molar amounts of reagent were used.

The starches were crosslinked with STMP (0.055%) both in the absence and in the presence of sodium sulfate (SS) (5 g/115 g H_2O). For normal maize starch, the product from crosslinking with STMP in the presence of SS produced much lower peak and final viscosities (CL [Fig. 5 – 1]) than did that crosslinked with STMP without SS (CL [Fig. 5 – 2]) or that crosslinked with 0.05% of $POCl_3$ (CL0.05 [Fig. 2 – 1]), indicating an apparent more efficient crosslinking when SS was present. For normal maize starch crosslinked with STMP in the absence of SS, the order of peak and final viscosities was the same as for samples

reacted with $POCl_3$: Enz-CL > Enz = unmodified > CL > CL-Enz. Normal maize starch crosslinked with STMP in the presence of SS produced the same peak and final viscosity order (Enz = unmodified > Enz-CL > CL = CL-Enz) and slightly lower viscosities as compared with starch crosslinked with 0.1% $POCl_3$.

For waxy maize starch modified with STMP (0.055%) in the presence of SS (Fig. 6 – 1), the peak viscosity produced by Enz-CL was lower than that produced by unmodified or enzyme-treated starch, and that produced by CL was the same as CL-Enz. However, in the absence of SS (Fig. 6 – 2), the peak viscosity of Enz-CL was greater than that of the unmodified or enzyme-treated samples. Waxy maize starch crosslinked with STMP in the absence of SS gave an RVA profile similar to that of waxy maize starch crosslinked with 0.075% $POCl_3$ (CL0.075 [Fig. 3 – 2]). However, waxy maize starch crosslinked with STMP in the presence of SS (Fig. 6 – 1) produced a RVA profile similar to that of waxy corn starch crosslinked with 0.1% $POCl_3$ (CL0.1 [Fig. 3 – 3]). For both normal and waxy maize starch modified with STMP in the presence of SS, the RVA profiles of CL and CL-Enz were the same, indicating that proteinase treatment did not produce any change in the crosslinked starch.

Whether $POCl_3$ or STMP was used as the crosslinking reagent, the profiles of products made from normal and waxy maize starch via crosslinking without and before and after treatment with the proteinase were quite different from one another (Fig. 2 vs. Fig. 5 and Fig. 3 vs. Fig. 6). In every case, Enz-CL (both $POCl_3$ and STMP) maize starches had a lower pasting temperature and produced higher peak and final viscosities than CL alone or even unmodified starches, i.e., Enz-CL starches appeared to be less crosslinked. The effect of protein removal was more pronounced in normal maize starch, as compared to waxy maize starch.

The starches used in this work were commercial starches because we wanted to determine the effects of proteins on commercial starches, even though in other studies, we have found that much of the protein associated with channel and external granule surfaces of corn starches is removed during the commercial wet-milling process by proteolytic enzymes in the steepwater (unreported). The total amount of protein (surface plus matrix) in these starches was reported to be in the order normal maize (0.83%) > waxy maize (0.10%) > potato (0.05%) (Greenwood & Thomson, 1962). Yoshino, Hayashi, and Seguchi (2005) reported amounts of surface proteins in commercial starch granules as being 0.436% for normal maize starch, 0.284% for waxy maize starch, and 0.121% for potato starch. Although the reported protein contents from these two studies differ quantitatively, they are in the same order, and it is clear that the amounts of surface and total proteins differ from starch to starch. For this reason, protein hydrolysis before or after crosslinking could produce different results in different starches. Also, granule-bound protein–starch crosslinks may play a role. GBSS, the principal protein in the matrix of granules of normal maize starches, is not present in waxy starches (Preiss, 1991). Han and Hamaker (2002) found that protein was concentrated in the envelopes (ghosts) of gelatinized and swollen granules of potato, normal maize, and wheat starch granules. Only traces of protein were found in the ghosts of waxy maize and amylose-free potato starch granules, which finding they attributed to a lack of GBSS in these starches. They then confirmed the presence of GBSS in granule ghosts of corn and potato starches and attributed the greater fragility of gelatinized waxy maize and amylose-free potato starch granules to the lack of GBSS. Because GBSS is a matrix protein and there is evidence that the matrix is not accessed by thermolysin (Han & Hamaker, 2002), GBSS is not likely removed by treatment with thermolysin. In this work, proteinase treatment of crosslinked normal maize starch produced a greater change than did proteinase treatment of crosslinked waxy maize starch. Although waxy maize starch has at least as many, if not more, channels than does normal maize starch, removing protein did not affect the pasting profile as much as it did with normal maize starch.

Fig. 7 shows pasting profiles of potato starch, which has neither pores nor channels and less protein than the maize

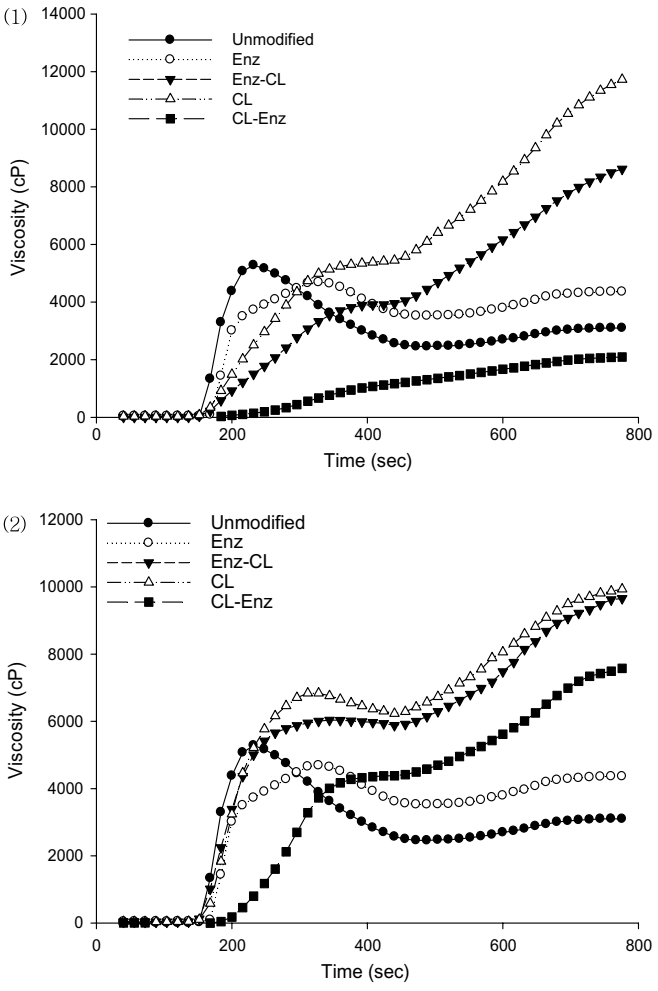

Fig. 7. RVA profiles of crosslinked (STMP) potato starch [(1) with SS (2) without SS)] before and after treatment with thermolysin.

starches, that was crosslinked with STMP in the presence and absence of SS. As with crosslinking with $POCl_3$, potato starch crosslinked with STMP behaved differently than did normal maize starch crosslinked with STMP. CL produced a higher final viscosity than did any other treatment in the presence of SS. (Final viscosities of CL and Enz-CL were about the same in the absence of SS.) A possible explanation is that the matrix of potato starch granules is less dense than that of maize starches, allowing more thorough reagent penetration into the granule matrix in potato starch as compared to the maize starches, but since potato starch contains much less protein than do the maize starches, protein–starch crosslinks are not indicated in this case.

4. Conclusions

To determine the effect of protein on crosslinking and whether protein–protein, protein–starch, and/or starch–starch crosslinking occurred when phosphoryl chloride was used as the crosslinking reagent, starch was reacted before and after treatment with a proteinase (thermolysin). It was concluded that chemical reaction may involve protein molecules.

The effect of removing protein could be seen most clearly with normal maize starch and crosslinking with $POCl_3$. Without protein removal, the apparent degree of crosslinking was much greater than it was when the protein was removed enzymically and the starch was reacted with the reagent in the same amounts and via the same procedure. Differences were apparent, but less pronounced, with waxy maize starch. After reaction with 0.10% $POCl_3$, native waxy corn starch was less inhibited than was either native or enzyme-treated normal corn starch reacted with 0.05% and 0.075% $POCl_3$. However, with waxy maize starch, as with normal maize starch, crosslinking following the enzyme treatment resulted in a product that was less inhibited than the same starch not treated with proteinase.

Results of crosslinking with STMP were basically the same as found with crosslinking with $POCl_3$, viz., crosslinking of normal maize starch appeared to be more effective than crosslinking of waxy maize starch, which contains less matrix protein than does normal maize starch (but it is not known if matrix protein plays a role), using the same amount of reagent, and removal of surface protein appeared to make the crosslinking reaction less effective. In addition, reactions with STMP in the presence of sodium sulfate appeared to be more effective than reactions carried out without sodium sulfate, with the difference being more pronounced with normal corn starch.

Potato starch crosslinked with $POCl_3$ gave different RVA profiles than did the two maize starches. However, the basic effects were the same as those found with the maize starches, i.e., reaction of potato starch with $POCl_3$ before surface protein removal appeared to be more effective in terms of crosslinking than was reaction of potato starch with $POCl_3$ after removal of surface protein, with the differences being more evident at the highest level of reagent use. Results from the crosslinking reaction of potato starch with STMP were also similar to those of the maize starches, viz., the reaction appeared to be more effective when sodium sulfate was present in the reaction mixture and less effective when surface protein was removed, the difference in the RVA profiles of products made by crosslinking in the absence of sodium sulfate with and without a prior treatment with thermolysin being slight.

References

Derycke, V., Veraverbeke, W. S., Vandeputte, G. E., De Man, W., Hoseney, R. C., & Delcour, J. A. (2005). Impact of proteins on pasting and cooking properties of nonparboiled and parboiled rice. *Cereal Chemistry, 82*, 468–474.

Fannon, J. E., Gray, J. A., Gunawan, N., Huber, K. C., & BeMiller, J. N. (2003). The channels of starch granules. *Food Science and Biotechnology, 12*, 700–704.

Fannon, J. E., Gray, J. A., Gunawan, N., Huber, K. C., & BeMiller, J. N. (2004). Heterogeneity of starch granules and the effect of granule channelization on starch modification. *Cellulose, 11*, 247–254.

Fannon, J. E., Hauber, R. J., & BeMiller, J. N. (1992). Surface pores of starch granules. *Cereal Chemistry, 69*, 284–288.

Gillian, D. A., Sargeant, J. G., & Schofield, J. D. (1981). Wheat starch granule proteins: The isolation and characterization of a salt-extractable protein from starch granules. *Journal of the Science of Food and Agriculture, 32*, 371–377.

Gray, J. A., & BeMiller, J. N. (2004). Development and utilization of reflectance confocal laser scanning microscopy to locate reaction sites in modified starch granules. *Cereal Chemistry, 81*, 278–286.

Greenwood, C. T., & Thomson, J. (1962). Physiochemical studies in starches. XXIV. Fractionation and characterisation of starches of various plant origins. *Journal of The Chemical Society*, 222–229.

Han, J.-A., Gray, J. A., Huber, K. C., & BeMiller, J. N. (2006). Derivatization of starch granules as influenced by the presence of channels and reaction conditions. In M. L. Fishman, P. X. Qi, & L. Wicker (Eds.). *Advances in biopolymers: Molecules, clusters, networks and interactions* (pp. 165–184). Washington, DC: American Chemical Society.

Han, X. Z., Benmoussa, M., Gray, J. A., BeMiller, J. N., & Hamaker, B. R. (2005). Detection of proteins in starch granule channels. *Cereal Chemistry, 82*, 339–351.

Han, X.-Z., & Hamaker, B. R. (2002). Location of starch granule-associated proteins revealed by confocal laser scanning microscopy. *Journal of Cereal Science, 35*, 109–116.

Hirsch, J. B., & Kokini, J. L. (2002). Understanding the mechanism of cross-linking agent ($POCl_3$, STMP, and EPI) through swelling behavior and pasting properties of cross-linked waxy maize starches. *Cereal Chemistry, 79*, 101–107.

Huber, K. C., & BeMiller, J. N. (2001). Location of sites of reaction within starch granules. *Cereal Chemistry, 78*, 173–180.

Lim, S.-T., Lee, J.-H., Shin, D.-H., & Lim, H. S. (1999). Comparison of protein extraction solutions for rice starch isolation and effects of residual protein content on starch pasting properties. *Starch/Stärke, 51*, 120–125.

Lim, S. T., & Seib, P. A. (1993). Preparation and pasting properties of wheat and corn starch phosphates. *Cereal Chemistry, 70*, 137–144.

Mu-Forster, C., Huang, R., Powers, J. R., Harriman, R. W., Knight, M., Singletary, G. W., et al. (1996). Physical association of starch biosynthetic enzymes with starch granules of maize endosperm: Granule-associated forms of starch synthase I and starch branching enzyme II. *Plant Physiology, 111*, 821–829.

Mu-Forster, C., & Wasserman, B. P. (1998). Surface location of zein storage proteins in starch granules from maize endosperm: Proteolytic

removal by thermolysin and in vitro cross-linking of granule associated polypeptides. *Plant Physiology, 116*, 1563–1571.

Nakamura, T., Yamamori, M., Hirano, H., & Hidaka, S. (1993). The waxy (Wx) proteins of maize, rice and barley. *Phytochemistry, 33*, 749–753.

Preiss, J. (1991). Biology and molecular biology of starch synthesis and its regulation. In B. J. Mifflin (Ed.). *Oxford surveys of plant molecular and cell biology* (Vol. 7, pp. 59–114). Oxford, UK: Oxford University Press.

Sano, Y. (1984). Differential regulation of waxy gene expression in rice endosperm. *Theoretical and Applied Genetics, 68*, 467–473.

Sigma–Aldrich Chemical Co. (1999). Thermolysin. www.Sigma–Aldrich.-com/SAcatalog.Nsf.

Tester, R. F., Yousuf, R., Kettlitz, B., & Röper, H. (2007). Use of commercial protease preparations to reduce protein and lipid content of maize starch. *Food Chemistry, 105*, 926–931.

Wood, T. M. (1994). Enzymic conversion of cellulose into D-glucose. *Methods in Carbohydrate Chemistry, 10*, 219–229.

Yoshino, Y., Hayashi, M., & Seguchi, M. (2005). Presence and amounts of starch granule surface proteins in various starches. *Cereal Chemistry, 82*, 739–742.