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# Effects of α-Amylases from Different Sources on the Firming of Concentrated Wheat Starch Gels: Relationship to Bread Staling

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The firming and carbohydrate fractions of concentrated starch gels supplemented with four α-amylases from different sources were evaluated. Correlations were found between the firmness data and results for the carbohydrate fractions extracted from the gels. The thermostable (TBA) and intermediate temperature stability (ISBA) bacterial α-amylases were most effective in decreasing the rate of firming. The cereal α-amylase at the high level (CAH) was also effective. The CAH produced the largest quantity of dextrins at storage time zero and the thermostable bacterial α-amylase at the high level (TBAH) after storage for 5 days. None of the maltooligosaccharides appeared to be responsible for the decreased rate of firming of the gels. The results indicated that the TBA and ISBA most effectively inhibited firming because they degraded the external branches and the intercluster regions of amylopectin during storage. Consideration of previously reported differential scanning calorimetry and X-ray crystallography results leads to the conclusion that the antifirming action of the TBA and ISBA is due to their ability to degrade the amylopectin and amorphous regions of the gels during storage, which inhibits the formation of double helices and decreases the strength of the starch gel matrix. Gels supplemented with the TBA and ISBA were most crystalline but firmed to a lesser extent. These results are similar to those previously reported by other researchers for bread and strongly suggest that starch retrogradation plays a primary role in bread staling.

**KEYWORDS: Amylopectin; amylases; bread staling; carbohydrates; crystallinity; degradation; dextrins; elastic modulus; firmness; gels; maltooligosaccharides; retrogradation; starch**

## INTRODUCTION

The current literature and theories on the effects of α-amylases on bread staling were discussed in previous papers (*1*, *2*). The results of our work on the characterization and comparisons of the carbohydrate fractions produced by the different α-amylase sources used to supplement the starch gels have been previously reported (*1*). The present work is the fourth of a series of experiments designed to characterize the carbohydrate fractions produced by α-amylases in concentrated starch gels and to relate these findings to the retrogradation and firming properties of these gels and bread.

The objectives of this study were (1) to compare the effects of different α-amylase sources on the firming of concentrated wheat starch gels, (2) to relate firmness data to the results obtained for the carbohydrate fractions extracted from these gels, and (3) to relate and discuss these findings in terms of bread staling.

## MATERIALS AND METHODS

**Materials.** The native wheat starch, enzymes, and maltooligosaccharide standards used in this study were previously described (*1*). The four α-amylases [(1→4)-α-D-glucan-glucanohydrolase; EC 3.2.1.1] used were of commercial origin. Thermostable bacterial α-amylase (TBA, 200 SKB units/mg) from *Bacillus subtilis* was from Serva (Paramus, NJ). Novamyl 1500 MG, a type II intermediate temperature stability bacterial α-amylase (ISBA, 1453 MANU/g), was from Novo Nordisk BioChem North America, Inc. (Franklinton, NC). Cereal α-amylase (CA, 445 SKB units/g after purification) from barley malt was from Sigma Chemical Co. (St. Louis, MO). Fungamyl 2500 BG, a fungal α-amylase (FA, 75 SKB units/mg) from *Aspergillus oryzae*, was from Novo Nordisk Bioindustrials, Inc. (Danbury, CT). Enzymes were purified and assayed as previously described (*1*). All additions were on a dry basis.

**Preparation of Starch Gels for Firmness Evaluation.** Gels larger than those previously used for differential scanning calorimetry (DSC) were required for firmness testing (*1*). The larger gels could not be prepared with the same heating schedule as previously described, as this would have resulted in uneven, not fully gelatinized gels. Therefore, it was necessary to adjust the enzyme levels to compensate for the different heating schedule. To determine the levels, starch gels containing the enzymes at several levels were prepared, extracted, and analyzed as described below. At 60 min following removal from the oven, the gels were frozen and freeze-dried. The freeze-dried gels were ground and extracted. The maltooligosaccharide extracts were analyzed using high-pressure anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), and the chromatograms were compared with the chromatograms of maltooligosaccharide extracted at storage time zero from gels previously prepared for enthalpy of

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**Table 1.** Effect of α-Amylases on the Elastic Modulus (*E*) of Starch Gels after Storage for Different Time Periods

| | $E^a$ (Pa × $10^4$) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| amylase | day 0 | day 1 | day 2 | day 3 | day 5 | day 8 | day 12 | day 20 | day 65 |
| TBAH | 81 ± 6 cd | 204 ± 14 e | 223 ± 20 f | 204 ± 13 e | 201 ± 9 f | 184 ± 13 d | 171 ± 14 e | 182 ± 5 e | 186 ± 8 e |
| ISBAH | 82 ± 5 cd | 179 ± 10 e | 215 ± 06 f | 236 ± 3 e | 264 ± 7 e | 288 ± 13 c | 310 ± 10 d | 314 ± 10 d | 325 ± 12 d |
| CAH | 68 ± 5 e | 251 ± 15 d | 308 ± 16 e | 365 ± 11 d | 413 ± 17 d | 464 ± 14 b | 496 ± 22 c | 517 ± 22 c | 564 ± 17 c |
| FAH | 86 ± 6 bc | 327 ± 22 bc | 409 ± 18 bd | 449 ± 16 c | 485 ± 16 bc | 521 ± 23 a | 549 ± 12 ab | 577 ± 17 ab | 645 ± 22 ab |
| TBAL | 85 ± 4 c | 344 ± 11 ab | 427 ± 19 ac | 448 ± 12 c | 467 ± 16 c | 477 ± 12 b | 512 ± 19 bc | 512 ± 13 c | 535 ± 17 c |
| ISBAL | 94 ± 2 ab | 308 ± 7 c | 389 ± 13 d | 446 ± 21 c | 478 ± 6 c | 518 ± 24 a | 534 ± 16 ab | 555 ± 24 b | 612 ± 19 b |
| CAL | 81 ± 2 cd | 309 ± 19 c | 397 ± 15 cd | 467 ± 18 bc | 513 ± 17 ab | 549 ± 20 a | 556 ± 21 a | 584 ± 16 ab | 657 ± 21 a |
| FAL | 84 ± 3 cd | 325 ± 06 bc | 431 ± 12 ab | 491 ± 09 ab | 519 ± 22 a | 546 ± 14 a | 558 ± 13 a | 588 ± 11 ab | 630 ± 23 ab |
| control | 95 ± 5 a | 361 ± 16 a | 452 ± 11 a | 504 ± 24 a | 521 ± 22 a | 550 ± 17 a | 562 ± 20 a | 596 ± 21 a | 655 ± 21 a |

[a] Means ± SD ($n = 5$). Means within a column followed by the same letter are not significantly different ($P < 0.05$).

retrogradation measurements (*1*). The low levels of the enzymes were selected to produce similar quantities of maltooligosaccharides as those produced by the low levels used in the preparation of gels previously described (*1*).

α-Amylases were added to starch at two levels of activity. For the low level the FA, CA, and TBA were added at 0.150, 0.070, and 0.010 SKB units/g of starch, respectively. The ISBA was added at 0.32 MANU/g of starch. The high level of activity corresponded to the low level multiplied by a factor of 10. The FA, CA, and TBA were added at 1.50, 0.70, and 0.10 SKB units/g of starch, respectively. The ISBA was added at 3.22 MANU/g of starch.

Enzymes were dissolved in 2 mM calcium chloride and added to 100.0 g of starch. Additional 2 mM calcium chloride was added to obtain 50% starch gels. A homogeneous starch slurry was prepared by thorough mixing with a spatula. The slurry was poured into tinplate containers (36 mm internal diameter), which had been sprayed with a thin layer of silicone release agent (316 silicone release spray, Dow Corning Corp., Midland, MI). Nine containers were filled with 15.0 g of slurry and tapped to remove air bubbles. The rims of the containers were wrapped with Teflon tape and the lids applied. To seal the containers, the seams were wrapped with high-temperature tape. The sealed containers were placed in a specially built rack and were tightly clamped to prevent the lids from popping. Thirty minutes elapsed from the point of addition of enzyme to the starch until the containers were placed in the oven. The containers were loaded in the oven of a gas chromatograph and heated to gelatinize the starch. The heating conditions were as follows: 50 °C for 6 min, 50−99 °C at a rate of 1 °C/min, and 99 °C for 5 min. The gels were removed from the oven and allowed to cool at room temperature for 60 min. After cooling, the containers were opened, and the gels were layered with a solution of 50 ppm of toluene in light mineral oil to prevent dehydration and microbial growth. The containers were resealed and stored at 20 °C for up to 65 days.

Eight α-amylase treatments (four α-amylase sources at two levels) and a control with no enzyme were prepared. Each treatment was replicated five times. One replicate consisted of nine starch gels, which were analyzed at storage time zero (60 min after removal from the oven) and after storage for 1, 2, 3, 5, 8, 12, 20, and 65 days.

**Firmness.** The elastic modulus (*E*) of the starch gels was determined using an Instron universal testing machine (Instron, Canton, MA) equipped with a 15-mm diameter plunger and a 5000-g load cell. Crosshead speed was set at 4 mm/min. The gels were removed from their containers, inverted, and compressed until rupture. The elastic modulus was determined by calculating the initial slope of the stress−strain curves. After analysis, cross sections of the gels (~3 g) were inserted into polypropylene vials fitted with screw caps and immediately frozen at −80 °C.

**Extraction of Starch Gels and Characterization of Extracts.** Extraction of starch gels, determination of total carbohydrate of the high molecular weight dextrin extracts, and HPAEC-PAD of malto-oligosaccharide extracts were performed as previously described (*1*).

**Statistical Analyses.** Firmness data were first analyzed using nonlinear regression to fit an Avrami equation to each of the five replicates (*3*−*6*). The equation was

$$E_t = E_L + (E_0 - E_L)\, e^{-kt^n} \quad (1)$$

where $E_0$, $E_t$, and $E_L$ are experimental values of the elastic modulus at time zero, $t$, and infinity, respectively, $k$ is a rate constant, $t$ is the independent variable time, and $n$ is the Avrami exponent (*7*, *8*). The parameters ($E_0$, $E_L$, $k$, and $n$) of the independent variable were estimated using the Marquardt−Levenberg algorithm according to SigmaStat 2.0 (Jandel Corp., San Rafael, CA). Despite its widespread use in cereal science, the Avrami theory does not seem to be a viable model to describe the mechanism of starch crystallization. Slade and Levine (*9*) have pointed out that the nonequilibrium nature of starch recrystallization limits the theoretical utility of the Avrami parameters. This suggestion has received support from other investigators (*10*, *11*). Regardless of these limitations, Slade and Levine (*9*) have pointed out that the Avrami equation can be used as a convenient method of fitting experimental data from starch-firming studies. In the present study, the Avrami equation has been used to fit the elastic modulus data. No inferences have been made as to the meaning of the Avrami parameters in the mechanistic and kinetic aspects of starch firming. The parameters $E_0$, $E_L$, and $k$ of the independent variable, the net limiting elastic modulus ($E_L - E_0$), $E_t$, and the linear rate of firming between storage time zero and day 5 ($LRF_{0/5}$) were analyzed using analysis of variance (ANOVA) according to a completely randomized design (CRD). Multiple comparisons of the treatment means were conducted using Tukey's test. At each storage time the quantities of individual maltooligosaccharides (glucose through maltododecaose), low molecular weight dextrins (LMWD), high molecular weight dextrins (HMWD), and total dextrins (TD) produced by the α-amylase treatments were analyzed using ANOVA and Tukey's test. A logarithmic transformation ($\log_{10}$) was applied to these data to meet the constant variance hypothesis required for ANOVA. Correlations between the data obtained for the carbohydrate analyses and the firming data were determined. All statistical analyses were performed using SigmaStat 2.0 (Jandel Corp.).

## RESULTS

**Firming of Starch Gels.** The effects of the different α-amylase sources and levels on the firming of starch gels are shown in **Table 1** and **Figure 1**. In general, the firming of the gels was characterized by a rapid increase in elastic modulus during the first 5 days of storage, a moderate increase from day 5 to day 20, and a slow increase thereafter. The firmness of gels supplemented with thermostable bacterial α-amylase at the high level (TBAH) increased during the first 2 days of storage and remained constant or slightly decreased thereafter (**Figure 1**). The kinetic parameters ($E_0$, $E_L$, $E_L - E_0$, $k$, and $n$) and plots obtained by fitting the elastic modulus data by an Avrami equation are shown in **Table 2** and **Figure 1**. Gels supplemented with CA exhibited the lowest values of $E_0$. The TBAH most effectively decreased the limiting elastic modulus

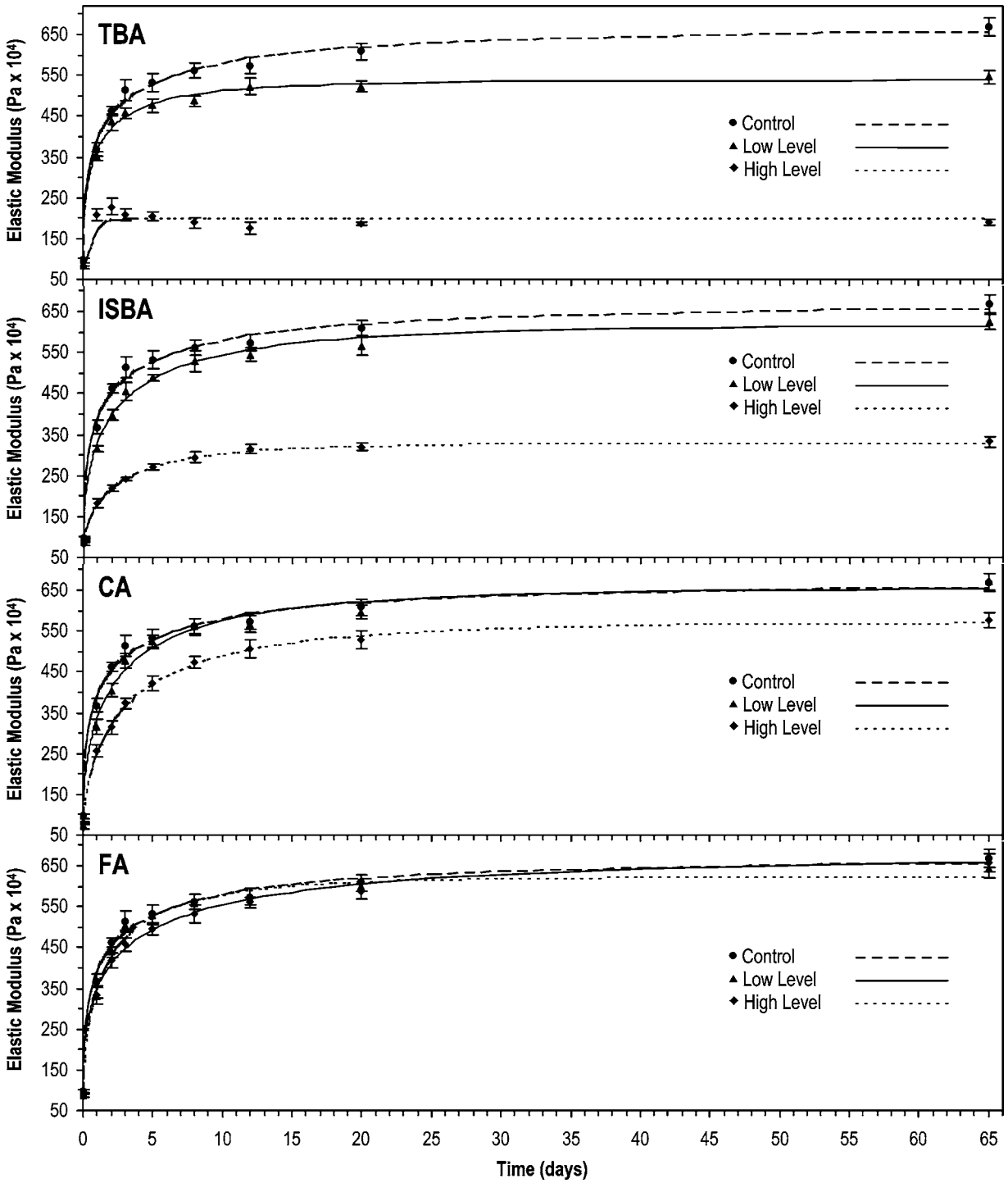


**Figure 1.** Effect of α-amylases from different sources on the kinetics of development of the elastic modulus in starch gels. Curves were fitted using an Avrami equation. Bullets represent actual data. Error bars represent ± 1 standard deviation ($n = 5$). TBA, thermostable bacterial α-amylase; ISBA, intermediate temperature stability bacterial α-amylase; CA, cereal α-amylase; FA, fungal α-amylase; Control, no enzyme added.

($E_L$) of the gels followed by the intermediate temperature stability bacterial α-amylase at the high level (ISBAH). Both the cereal α-amylase at the high level (CAH) and the thermostable bacterial α-amylase at the low level (TBAL) were effective in decreasing the $E_L$ of the gels but not as effective as the TBAH and ISBAH. The $E_L$ of gels supplemented with intermediate temperature stability bacterial α-amylase at the low level (ISBAL) was not significantly different from that of the unsupplemented control gels, but the elastic moduli of these gels were consistently lower than those of the control gels at every storage time (**Figure 1**). The values of $E_L$ of gels supplemented with each of the remaining α-amylases were not significantly different from that of the unsupplemented control gels. The values of $E_L - E_0$ ranked in the same order as the values of $E_L$. The $k$ of gels supplemented with TBAH was significantly higher than those for the other treatments. To a lesser extent, the $k$ of gels supplemented with TBAL was also significantly higher than those for the other treatments. The values of $k$ of gels supplemented with ISBAH or CAH were the lowest. Except for gels supplemented with TBAH, the values of $n$ for all treatments were smaller than unity. The kinetic parameters ($E_0$, $E_L$, $E_L - E_0$, and $k$) obtained by fitting the elastic

**Table 2.** Kinetic Parameters Calculated from Elastic Modulus Data Obtained from Starch Gels Supplemented with α-Amylases

| | parameters[a] | | | | |
|---|---|---|---|---|---|
| amylase | $E_L$ (Pa × $10^4$) | $E_0$ (Pa × $10^4$) | $E_L - E_0$ (Pa × $10^4$) | $k^b$ ($day^{-n}$) | $n$ |
| TBAH | 190 ± 10 e | 85 ± 1 bc | 111 ± 1 e | 1.37 ± 0.06 a | 1.50 ± 0.01 a |
| ISBAH | 325 ± 13 d | 81 ± 5 c | 243 ± 9 d | 0.51 ± 0.01 e | 0.64 ± 0.02 b |
| CAH | 563 ± 29 bc | 68 ± 5 d | 495 ± 26 bc | 0.46 ± 0.01 e | 0.59 ± 0.03 bc |
| FAH | 664 ± 58 a | 85 ± 6 bc | 579 ± 52 a | 0.59 ± 0.02 d | 0.42 ± 0.07 ef |
| TBAL | 528 ± 17 c | 84 ± 4 bc | 443 ± 13 c | 0.96 ± 0.03 b | 0.47 ± 0.04 df |
| ISBAL | 606 ± 43 ab | 92 ± 2 ab | 513 ± 41 ab | 0.60 ± 0.03 d | 0.52 ± 0.05 ce |
| CAL | 643 ± 49 a | 77 ± 2 c | 565 ± 46 a | 0.59 ± 0.04 d | 0.52 ± 0.05 cd |
| FAL | 613 ± 26 ab | 81 ± 2 c | 531 ± 25 ab | 0.70 ± 0.01 c | 0.55 ± 0.04 bd |
| control | 656 ± 46 a | 94 ± 6 a | 562 ± 41 a | 0.73 ± 0.05 c | 0.41 ± 0.03 f |

[a] Means ± SD ($n = 5$). Means within a column followed by the same letter are not significantly different ($P < 0.05$). [b] Units of $k$ depend on the value of the Avrami exponent ($n$).

**Table 3.** Kinetic Parameters with Avrami Exponent Set as Unity ($n = 1$) Calculated from Elastic Modulus Data Obtained from Starch Gels Supplemented with α-Amylases

| | parameters[a] | | | |
|---|---|---|---|---|
| amylase | $E_{L,n=1}$ (Pa × $10^4$) | $E_{0,n=1}$ (Pa × $10^4$) | $(E_L - E_0)_{n=1}$ (Pa × $10^4$) | $k_{n=1}$ ($day^{-1}$) |
| TBAH | 188 ± 11 e | 81 ± 6 d | 112 ±05 f | 9.64 ± 0.86 a |
| ISBAH | 312 ± 12 d | 93 ± 7 cd | 219 ±05 e | 0.37 ± 0.02 b |
| CAH | 521 ± 22 bc | 95 ± 9 bc | 426 ± 15 cd | 0.32 ± 0.01 b |
| FAH | 569 ± 18 a | 108 ± 5 ab | 462 ± 13 ac | 0.49 ± 0.06 b |
| TBAL | 500 ± 15 c | 90 ± 4 cd | 410 ± 11 d | 0.87 ± 0.03 b |
| ISBAL | 554 ± 25 ab | 110 ± 4 a | 443 ± 21 bd | 0.48 ± 0.02 b |
| CAL | 587 ± 44 a | 96 ± 9 bc | 491 ± 36 a | 0.47 ± 0.05 b |
| FAL | 576 ± 18 a | 92 ± 3 cd | 484 ± 15 a | 0.59 ± 0.01 b |
| control | 582 ± 30 a | 107 ± 9 ab | 475 ± 21 ab | 0.65 ± 0.06 b |

[a] Means ± SD ($n = 5$). Means within a column followed by the same letter are not significantly different ($P < 0.05$).

**Table 4.** Linear Rate of Firming between Days 0 and 5 ($LRF_{0/5}$) and Elastic Modulus ($E$) at Days 0 and 5 of Starch Gels Supplemented with α-Amylases[a]

| amylase | $LRF_{0/5}$ (Pa × $10^4$/day) | $E$, day 0 (Pa × $10^4$) | $E$, day 5 (Pa × $10^4$) |
|---|---|---|---|
| TBAH | 24 ± 1 f | 81 ± 6 c | 201 ± 10 f |
| ISBAH | 36 ± 1 e | 81 ± 5 c | 265 ±07 e |
| CAH | 69 ± 3 d | 69 ± 5 d | 414 ± 17 d |
| FAH | 79 ± 2 bc | 86 ± 6 bc | 485 ± 16 bc |
| TBAL | 76 ± 3 c | 85 ± 4 c | 466 ± 16 c |
| ISBAL | 76 ± 1 c | 94 ± 2 ab | 478 ± 6 c |
| CAL | 86 ± 4 a | 80 ± 2 c | 514 ± 18 ab |
| FAL | 87 ± 4 a | 83 ± 3 c | 519 ± 22 a |
| control | 85 ± 3 ab | 95 ± 6 a | 521 ± 22 a |

[a] Means ± standard deviation ($n = 5$). Means within a column followed by the same letter are not significantly different ($P < 0.05$).

modulus data by an Avrami equation, with $n$ constrained to be unity, are shown in **Table 3**. In general, use of this model increased the values of $E_0$ and decreased the values of $E_L$ and $E_L - E_0$. The values of $k$ were increased or decreased depending on the enzyme treatment. Most notably, the $k$ of gels supplemented with TBAH was increased by a factor of 6 when this model was applied.

**Linear Rate of Firming.** The linear rates of firming between storage time zero and day 5 ($LRF_{0/5}$) for gels supplemented with the α-amylases are summarized in **Table 4**. These rates were calculated to compare with the X-ray crystallography data obtained from gels stored for 5 days (*2*). Among the gels supplemented with the high levels, those supplemented with TBAH or ISBAH exhibited the lowest rates of firming. Gels supplemented with CAH also exhibited rates of firming that were significantly lower than that of the unsupplemented control gels. The rate of firming of gels supplemented with fungal α-amylase at the high level (FAH) was not different from that of the unsupplemented control gels. Among the gels supplemented with the low levels, those supplemented with TBAL or ISBAL were the only ones that exhibited a rate of firming that was significantly lower than that of the unsupplemented control gels. The rates of firming of gels supplemented with cereal α-amylase at the low level (CAL) or fungal α-amylase at the low level (FAL) did not differ from that of the unsupplemented control gels.

**Carbohydrate Extracts.** The heating schedule used to prepare gels for firmness measurements was different from that previously used to prepare gels for DSC measurements (*1*, *2*). Therefore, in the current study supplementation with the low levels of the enzymes was adjusted to give similar levels of LMWD. The low level of TBA was not changed because this enzyme produced the majority of the LMWD during storage. However, the low level of ISBA was reduced by a factor of 2. The low levels of both the CA and FA were reduced by a factor of 3. The high levels were previously obtained by increasing the low levels by a factor of 25 (*1*), whereas in the current experiments the high levels were obtained by increasing the low levels by a factor of 10. The reason for this difference was that use of a factor of 25 in these experiments gave gels that were too soft and very difficult to handle. The results obtained for individual maltooligosaccharides (glucose through maltododecaose) in the current study are not shown because they were similar to those previously reported for the gels prepared for DSC determinations (*1*). The profiles of maltooligosaccharides and the relative rankings for the individual maltooligosaccharides, LMWD, HMWD, and TD extracted from gels supplemented with the different α-amylases were also very similar. However, the quantities of maltooligosaccharides produced by the high levels of the enzymes used in these experiments were lower. Therefore, the data previously presented (*1*) should also be valid for these gels. For comparison purposes, the results obtained for the LMWD and HMWD extracted from starch gels at storage time zero and after storage for 5 days are summarized in **Table 5**. These results are not further discussed here in terms of the products and degradation produced by each of the α-amylases because an in-depth discussion was previously presented (*1*). However, they are discussed here in terms of their relationship to firmness of the starch gels.

**Table 5.** Low and High Molecular Weight Dextrins Extracted at Storage Times Day 0 and Day 5 from Starch Gels Supplemented with α-Amylases[a]

| | LMWD (mg/g of solids) | | HMWD (mg/g of solids) | |
|---|---|---|---|---|
| amylase | day 0 | day 5 | day 0 | day 5 |
| TBAH | 54.1 ± 2.1 d | 326.9 ± 14.8 a | 295.2 ± 4.7 a | 226.6 ± 6.8 a |
| ISBAH | 92.8 ± 3.0 b | 155.4 ±06.1 b | 81.5 ± 4.5 e | 136.8 ± 5.7 b |
| CAH | 172.3 ± 3.4 a | 137.6 ±02.2 c | 189.6 ± 5.0 b | 123.4 ± 2.5 c |
| FAH | 62.2 ± 1.4 c | 42.3 ±01.9 d | 64.4 ± 4.1 g | 40.9 ± 2.6 e |
| TBAL | 4.8 ± 0.2 f | 23.4 ±00.4 f | 74.6 ± 2.3 f | 36.2 ± 2.9 e |
| ISBAL | 21.5 ± 1.3 e | 29.3 ±02.4 e | 62.1 ± 3.0 g | 32.1 ± 1.2 ef |
| CAL | 22.6 ± 0.4 e | 17.7 ±00.3 g | 135.2 ± 2.4 c | 83.3 ± 1.4 d |
| FAL | 19.9 ± 0.2 e | 15.9 ±00.3 h | 99.2 ± 1.9 d | 29.5 ± 0.6 f |
| control | 2.1 ± 0.1 g | 2.2 ± 00.1 i | 79.6 ± 2.9 ef | 16.0 ± 0.5 g |

[a] Means ± SD ($n = 5$). Means within a column followed by the same letter are not significantly different ($P < 0.05$). A log transformation was applied to these data to meet the constant variance hypothesis required for ANOVA; the data shown are untransformed.

## DISCUSSION

The following discussion integrates the results obtained for the carbohydrate fractions extracted from these gels (*1*) with the firmness results reported above. The results on firming indicated that the starch gels supplemented with TBA, ISBAH, or CAH had significantly lower values of $E_L - E_0$ than the unsupplemented control gels. In contrast, the values of $E_L - E_0$ of gels supplemented with the remaining α-amylases (FA, ISBAL, or CAL) did not differ from that of the unsupplemented control gels. Even though the $E_L - E_0$ of gels supplemented with ISBAL was not significantly different from that of the control, the $LRF_{0/5}$ of these gels was significantly lower than that of the control. Correlation coefficients ($r$) between $E_L - E_0$ and the quantities of individual maltooligosaccharides (glucose through maltododecaose) extracted at storage time zero and after storage for 1, 2, 3, 5, 8, 12, 20, and 65 days are presented in **Table 6**. The only maltooligosaccharides that were significantly correlated with $E_L - E_0$ were glucose, maltose, maltotriose, and maltotetraose. The negative sign of these correlations appears to indicate that gels that contained the largest quantities of these maltooligosaccharides firmed to a lesser extent. However, these findings should be viewed with caution. The negative correlations do not necessarily mean that these maltooligosaccharides were responsible for the decrease in $E_L - E_0$. The quantities of both glucose and maltose continually increased during storage in gels supplemented with TBA or ISBA because these enzymes remained partially active and continually degraded the starch and dextrins (*1*). As it was previously reported (*1*), the TBA degraded maltooligosaccharides larger than maltohexaose into smaller maltooligosaccharides, whereas the ISBA degraded maltooligosaccharides as small as maltotriose into glucose and maltose. In fact, the strength and significance of the correlations between $E_L - E_0$ and maltotriose and maltotetraose decreased as the storage time progressed. The reason for this is that the ISBA, which effectively decreased $E_L - E_0$, especially at the high level, degraded these two maltooligosaccharides during storage (*1*). The opposite can be said for glucose and maltose. The strength and significance of the correlations with $E_L - E_0$ for these two sugars increased during storage because the TBA and ISBA, which most effectively decreased $E_L - E_0$, continually produced them. The finding that none of the maltooligosaccharides larger than maltotetraose was significantly correlated with $E_L - E_0$ appears to indicate that the maltooligosaccharides were not the direct cause of the reduced values of $E_L - E_0$ in gels supplemented with TBA, ISBAH, or CAH. Gels supplemented with CAH contained larger quantities of maltooligosaccharides larger than maltotetraose at storage time zero than gels supplemented with either TBAH or ISBAH but had an $E_L - E_0$ that was significantly larger than those of gels supplemented with TBAH or ISBAH. Similarly, gels supplemented with CAH contained much greater quantities of maltooligosaccharides larger than maltotriose after storage for 5 days but had an $E_L - E_0$ significantly higher than that of gels supplemented with ISBAH. Gels supplemented with FAH had an $E_L - E_0$ that did not differ from that of the unsupplemented control gels. However, these gels contained larger quantities of maltooligosaccharides than gels supplemented with TBAL, which had an $E_L - E_0$ that was significantly lower than that of the control. These results suggest that there is no direct relationship between the quantities of individual maltooligosaccharides and the firmness of the starch gels. Furthermore, the finding that the TBA and ISBA were the most effective enzymes in decreasing $E_L - E_0$ and remained partially active in the gels indicates that the residual activity plays an important role in the reduction of $E_L - E_0$. In fact, it appears that degradation of the starch gel matrix during storage more effectively decreased $E_L - E_0$ than an extensive degree of starch degradation during starch gelatinization. This is supported by the observation that the CAH produced the largest quantities of maltooligosaccharides during starch gelatinization but was not as effective in decreasing $E_L - E_0$ as the TBAH and ISBAH, which produced lower amounts of maltooligosaccharides during the preparation of the gels.

**Table 6.** Correlation Coefficients ($r$) between the Net Limiting Elastic Modulus and Maltooligosaccharides Extracted at Different Storage Times from Starch Gels Supplemented with α-Amylases[a]

| maltooligo-saccharide | day 0 | day 1 | day 2 | day 3 | day 5 | day 8 | day 12 | day 20 | day 65 |
|---|---|---|---|---|---|---|---|---|---|
| G1 | ns | ns | –0.64* | –0.68* | –0.76* | –0.83** | –0.89** | –0.89** | –0.83** |
| G2 | –0.65* | –0.67* | –0.70* | –0.70* | –0.71* | –0.73* | –0.72* | –0.73* | –0.71* |
| G3 | ns | –0.78* | –0.77* | –0.75* | –0.75* | –0.73* | –0.73* | –0.71* | –0.66* |
| G4 | ns | –0.84** | –0.86** | –0.80** | –0.69* | –0.67* | ns | ns | ns |
| G5 | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| G6 | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| G7 | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| G8 | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| G9 | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| G10 | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| G11 | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| G12 | ns | ns | ns | ns | ns | ns | ns | ns | ns |

[a] ns, not significant; *, significant at $P \leq 0.05$; **, significant at $P \leq 0.01$.

**Table 7.** Correlation Coefficients (*r*) of the Net Limiting Elastic Modulus with Dextrin Fractions Extracted at Different Storage Times from Starch Gels Supplemented with α-Amylases[a]

| dextrin fraction | day 0 | day 1 | day 2 | day 3 | day 5 | day 8 | day 12 | day 20 | day 65 |
|---|---|---|---|---|---|---|---|---|---|
| LMWD | ns | –0.89** | –0.92** | –0.93** | –0.94** | –0.95** | –0.96** | –0.98** | –0.97** |
| HMWD | –0.70* | –0.81** | –0.85** | –0.86** | –0.87** | –0.88** | –0.87** | –0.89** | –0.89** |
| TD[b] | –0.76* | –0.86** | –0.89** | –0.91** | –0.92** | –0.93** | –0.94** | –0.96** | –0.96** |

[a] ns, not significant; *, significant at $P \leq 0.05$; **, significant at $P \leq 0.01$. [b] TD, total dextrins (sum of LMWD and HMWD).

Therefore, the maltooligosaccharides appeared to be just an expression of the degree of starch modification produced by the α-amylases.

The correlation coefficients between $E_L - E_0$ and the carbohydrate fractions extracted from the gels are presented in **Table 7**. The LMWD, HMWD, and TD extracted from the gels were negatively correlated with $E_L - E_0$. The finding that none of the individual maltooligosaccharides larger than maltotetraose were significantly correlated with $E_L - E_0$ but were significantly correlated when considered as a group in the LMWD lends support to the suggestion that their presence is just an expression of structural changes in the starch. The negative sign of these correlations, plus their increase in strength and significance as the storage time progressed, suggests that the enzymes that were most effective in decreasing $E_L - E_0$ were those that produced the greatest quantities of dextrins (LMWD and HMWD) or starch degradation during storage.

The proportion of short branches with a degree of polymerization (DP) of 2−10 in the HMWD extracted after storage for 2 days from the starch gels supplemented with the high levels of the α-amylases and the control gels (*1*) was negatively correlated with $E_L - E_0$ ($r = -0.82$; $P \leq 0.05$), whereas the proportion of longer branches (DP = 21−30) was positively correlated ($r = 0.92$; $P \leq 0.01$). No significant correlation was found between the proportion of intermediate length branches (DP = 11−20) in the HMWD and $E_L - E_0$. Considering that the HMWD were branched fragments that originated from the degradation of amylopectin, it is clear that starch gels, in which the branches of amylopectin were shortened to a larger extent, firmed to a lesser extent. We previously suggested (*1*) that both the TBAH and ISBAH produced an extensive degradation of the external branches of amylopectin during storage of the gels, and this helps to explain why these two enzymes were most effective in inhibiting firming.

Even though the CAH was more effective in decreasing the $E_L - E_0$ of the starch gels than the FAH, the HMWD in gels supplemented with CAH contained lower proportions of short branches (DP = 2−10) than those in gels supplemented with FAH. A possible explanation for this inconsistency is that the overall degradation of the starch produced by the CAH was much more extensive than that produced by the FAH (*1*). Therefore, it is possible that the larger extent of starch degradation produced by the CAH more effectively inhibited starch retrogradation even though the proportion of short branches (DP = 2−10) in the HMWD produced by this enzyme was lower than that in the HMWD produced by the FAH.

We previously reported that the amylopectin in starch gels supplemented with TBA or ISBA was degraded to a larger extent than the amylopectin in the control gels and in gels supplemented with FA or CA (*1*). This was evidenced by a progressive decrease in the high molecular weight amylopectin fraction (AP I) and a progressive increase in the low molecular weight amylopectin fraction (AP II) during storage in the residual starches obtained from gels supplemented with TBA or ISBA. The decrease in size of the AP I molecules probably took place through degradation of intercluster regions. The peak corresponding to amylose (AM) also increased in gels supplemented with TBA or ISBA during storage due to the degradation of amylopectin into low molecular weight fragments, which coeluted with amylose. In the current study it was found that at storage time day 5 the proportion of AP I extracted from the starch gels was positively correlated with the $E_L - E_0$ of the gels ($r = 0.97$; $P \leq 0.01$), whereas the proportions of AP II ($r = -0.95$; $P \leq 0.01$) and the peak representing AM ($r = -0.92$; $P \leq 0.01$) were negatively correlated with $E_L - E_0$. Clearly, this indicates that the enzymes which were able to degrade amylopectin to a greater extent most effectively inhibited firming of the gels. These findings suggest that the ability to decrease the $E_L - E_0$ of the ISBA and TBA was due to the fact that these enzymes remained partially active in the gels and continually degraded the amylopectin fraction of starch during storage. The decrease in $E_L - E_0$ produced by the CAH can be explained on the basis that this enzyme extensively degraded the starch during preparation of the gels (*1*). However, the effects produced by the TBA and ISBA on $E_L - E_0$ were more drastic than that produced by the CAH. This also suggests that degradation of the starch gels during storage inhibits firming to a larger extent than an extensive initial degradation during gelatinization of the starch alone.

The *k* values of the gels supplemented with TBA were significantly higher than those of the other treatments. A possible explanation for this, especially for the gels supplemented with TBAH, is that the high values of *k* observed for these gels were a result of the low values of $E_L - E_0$ of these gels. The results in **Table 1** show that the firmness of gels supplemented with TBAH slowly decreased after the second day of storage, most probably due to the ability of this enzyme to remain partially active and degrade the starch gel network. The remaining treatments had values of *k* that were lower than those of the control.

The changes and effects produced by the α-amylases on the firming properties of the starch gels are similar to those reported by others for bread is supplemented with α-amylases. Both bacterial α-amylase (TBA) and intermediate temperature stability bacterial α-amylase (ISBA) have been shown to retard and/or reduce the firmness of bread (*12*−*14*). Therefore, the results obtained for the firmness of the starch gels supplemented with the bacterial enzymes are in agreement with those reported when they are used to supplement bread. Supplementation with FA did not significantly decrease the firmness of the starch gels. These findings agree with previous studies in which supplementation of bread with fungal α-amylase had little or no effect on the firmness of bread (*12*, *14*). Supplementation with CAH significantly decreased the firmness of the starch gels. These results also agree with previous results when supplementation of bread with malted barley flour effectively decreased the firmness (*12*, *15*, *16*). However, Martin and Hoseney (*13*) reported that bread supplemented with malted barley flour (CA)

firmed at a faster rate than unsupplemented bread. According to Martin and Hoseney (*13*), bread supplemented with malted barley flour firmed at a faster rate because it did not contain dextrins with a DP of 3−9. Martin and Hoseney contend that these dextrins interfere with gluten−starch interactions, to which they attributed the main role in the firming of bread crumb. They also suggested that starch−starch interactions (starch retrogradation) do not play a role in bread firming. This suggestion is partly based on results from studies in which the crystallinity of starch in bread supplemented with α-amylases, which firmed to a lesser extent than unsupplemented bread, was higher than that of starch in unsupplemented bread (*12*, *17*). However, the results obtained in the present study indicate that the effects of supplementation of starch gels with α-amylases are similar to those observed in bread. The correlation coefficient between the relative crystallinity (*2*) and the $LRF_{0/5}$ of the gels was −0.85 ($P \leq 0.01$). Therefore, the effect of the α-amylases was to decrease the firmness and to increase the crystallinity of the starch gels. Considering that in the starch gels the only possible interactions are starch−starch interactions and that the firmness−crystallinity relationship is analogous to that observed in bread, it is clear that this relationship is no basis to disregard the role of starch retrogradation in bread firming. Furthermore, we have previously shown that the main factor contributing to the retrogradation of starch in the gels supplemented with α-amylases is the degree of molecular order or double-helical content (*1*). In this respect, the effect of the α-amylases was to decrease the degree of molecular order in the gels. The correlation coefficient between the $E_L - E_0$ of the gels prepared for evaluation of firmness and the limiting enthalpy of retrogradation of the gels used for evaluation of the enthalpy of retrogradation (*1*) was 0.94 ($P \leq 0.01$). The fact that these two parameters of the gels were strongly correlated suggests that the antifirming effect of the α-amylases is a consequence of their ability to decrease the molecular order in the starch gels, or similarly in the starch fraction of bread as reported by Morgan et al. (*18*). Again, this lends support to the suggestion that the determining factor in the firming and retrogradation of starch gels or bread supplemented with α-amylases is the degree of molecular order or double-helical content of the starch. The results from the carbohydrate analyses indicate that the degradation of amylopectin (external branches and intercluster regions) by the α-amylases was the main factor that contributed to the decreases in the degree of molecular order and firmness of the starch gels. In this respect, the α-amylases that remained partially active in the gels during storage (TBA and ISBA) were most effective in inhibiting firming of the gels. Zobel and Senti (*17*) reported that bread supplemented with bacterial α-amylase firmed at a slower rate and was more crystalline than unsupplemented bread. On the basis of these results, Zobel and Senti (*17*) hypothesized that this α-amylase remained partially active in the bread and was able to degrade the amorphous regions of the starch gel in bread crumb. As a result of this degradation, the starch gel network lost rigidity and became more crystalline. The results in the present study and those we previously reported (*1*, *2*) support this hypothesis and suggest that the decrease in firmness is mainly due to the ability of the enzyme to decrease the double-helical content of the starch gel due to the degradation of amylopectin.

The results of the present study also indicate that the maltooligosaccharides (LMWD) produced by the enzymes do not appear to play a primary role in the decreases observed in the firmness of the starch gels. On the contrary, these dextrins appear to be an indicator of the extent of starch degradation, which is the cause of the inhibitory effect that certain α-amylases have on bread firming. These results agree with those reported for bread by Gerrard et al. (*19*), who suggested that the dextrins produced by α-amylases in bread are just an expression of a modification to starch, which is the causal factor in the inhibition of bread staling.

## ABBREVIATIONS USED

AM, amylose; AP I, high molecular weight amylopectin; AP II, low molecular weight amylopectin; CA, cereal α-amylase; CAH, cereal α-amylase at the high level; CAL, cereal α-amylase at the low level; CRD, completely randomized design; DP, degree of polymerization; DSC, differential scanning calorimetry; $E$, elastic modulus; $E_0$, elastic modulus at time zero; $E_L$, limiting elastic modulus; $E_L - E_0$, net limiting elastic modulus; FA, fungal α-amylase; FAH, fungal α-amylase at the high level; FAL, fungal α-amylase at the low level; HMWD, high molecular weight dextrins; HPAEC-PAD, high-pressure anion exchange chromatography with pulsed amperometric detection; ISBA, intermediate temperature stability bacterial α-amylase; ISBAH, intermediate temperature stability bacterial α-amylase at the high level; ISBAL, intermediate temperature stability bacterial α-amylase at the low level; ISBAM, intermediate temperature stability bacterial α-amylase at the medium level; $k$, rate constant, LMWD, low molecular weight dextrins; $LRF_{0/5}$, linear rate of firming between storage time zero and day 5; MANU, maltogenic amylase Novo units; $n$, Avrami exponent; TBA, thermostable bacterial α-amylase; TBAH, thermostable bacterial α-amylase at the high level; TBAL, thermostable bacterial α-amylase at the low level; TD, total dextrins.

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