FACTORS INVOLVED IN THE OXIDATIVE GELATION OF WATER-SOLUBLE PENTOSANS

by

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B.S., University of Arkansas, 1985

A THESIS

submitted in partial fulfillment
of the requirements for the degree

MASTER OF SCIENCE

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1988

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Pentosans, the nonstarchy and noncellulosic polysaccharides of plants, are produced in plants as structural components of the cell wall. Wheat flour contains both water-soluble and water-insoluble pentosans.

At room temperature and in the presence of certain oxidants, water-soluble pentosans form a gel. The mechanism for this oxidative gelation is not completely understood. The gelling ability of the water-soluble pentosans is believed to involve ferulic acid.

The objectives of this study were: (1) to determine factors contributing to gelation of aqueous extracts of flour, (2) to study the mechanism of oxidative gelation, and (3) to understand changes in the physical properties of aqueous extracts with time.

LITERATURE REVIEW

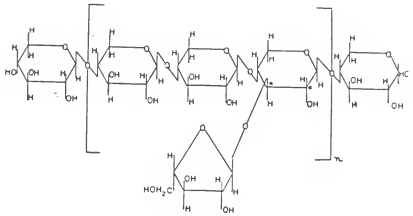
Wheat flour contains both water-soluble and water-insoluble pentosans. Aqueous extraction of flour separates water-insoluble pentosans (~2.4%) from water-soluble pentosans (~0.8%). The water-insoluble pentosan fraction is predominant in the tailings fraction. While the name infers polymers of pentose sugars, the pentosan fraction also contains hexoses, proteins, and phenolic acids.

Conflicting reports on the structure and composition of the water-soluble pentosans result from the various isolation and purification techniques employed. A single extraction of flour at a 2:1 to 4:1 ratio of water to flour has been commonly used to isolate the water-soluble fraction (Baker et al 1943, Kuendig et al 1961, Medcalf et al 1968, Montgomery and Smith 1955, Painter and Neukom 1968, Lineback et al 1977, Perlin 1951a, Yeh et al 1980). Extraction of flour with a 1:2 ratio of flour to water yields approximately 0.8% water-soluble pentosans, based on flour weight (Baker et al 1945). Multiple extractions of the same flour may be used to extract higher molecular weight and "difficult-to-dissolve" fractions more completely (Suckow et al 1983).

Structure

The chemical structure of the predominant water-soluble pentosan fraction was reported by Perlin (1951b, Fig. 1) to consist of a backbone of anhydro-D-xylopyranosyl residues linked B-(1-4) with single anhydro-L-arabinofuranosyl side chains at positions 2 or 3 of the xylose residues. These branches are responsible for solubility of

Figure 1. Structure of the water-soluble pentosans, reproduced from Perlin, 1951b.



the polymers as they prevent intermolecular aggregation of unsubstituted xylan residues (Andrewartha et al 1979). Removal of the side chains results in an insoluble xylan chain (Perlin 1951a).

Water-insoluble pentosans have a more highly branched structure than the water-soluble pentosans (Medcalf and Gilles 1968, Perlin 1951a, Perlin and Suzuki 1965, Montgomery and Smith 1955). Approximately 60% of the xylose residues are branched and almost half of these are branched at both positions 2 and 3 (Medcalf and Gilles 1968, Perlin 1951a). The insolubility of this fraction results from a high degree of intermolecular aggregation (Perlin 1951a). On the other hand, Suckow et al (1983) claim that the degree of polymerization (or molecular weight) accounts for the water-insolubility of this fraction.

Composition of water-solubles

Several studies used ion-exchange chromatography on diethylaminoethyl (DEAE)-cellulose column to fractionate the water solubles into five fractions (Kuendig et al 1961, Lineback et al 1977, MacArthur and D'Appolonia 1980, Medcalf et al 1968). The first fraction was identified as an arabinoxylan and the remaining four fractions were identified as glycoproteins. Disagreement existed concerning the actual number of different fractions. The controversy was resolved when Fincher et al (1974) showed that the pentosans consist of an arabinoxylan, a protein that is not covalently bound, and a protein that is covalently linked to a polypeptide. The water-soluble pentosans were separated into 2 fractions by either salting out with ammonium sulfate (Fincher et al 1974) or by precipitation

with 65% ethanol (Suckow et al 1983). With these two methods, the insoluble fraction was identified as an arabinoxylan and the insoluble fraction a peptide-bound arabinogalactan. The polysaccharide and peptide are presumably glycosidically linked through the hydroxyl group of hydroxyproline residues. Viscometric studies of flour-water extracts showed that the polysaccharide accounted for 95% of the intrinsic viscosity while 5% was due to soluble protein (Udy 1956). Ferulic acid (4-hydroxy-3-methoxycinnamic acid), a phenolic constituent of higher plant cell walls, is esterified to the largest arabinoxylan fraction (Yeh et al 1980).

Physical properties of water-soluble pentosans

Being highly branched gives pentosans unique physical properties. In water, pentosans form viscous solutions. Here viscosity is defined as resistance to flow. Viscosity is influenced by such factors as: molecular weight, shape, and concentration of the molecule.

The viscosity of aqueous wheat flour extracts is concentration dependent. For example: a 0.5% solution of water-solubles is described as mucilaginous while a 2% solution is difficult to pour, and a 4% solution forms a soft gel (Neukom et al 1967). Relative viscosity of aqueous extracts of wheat flour increases dramatically when treated with hydrogen peroxide (Durham 1925).

Oxidation of aqueous extracts

Concentrated water-soluble pentosan solutions form a gel at room temperature in the presence of oxidants. This unique property of oxidative gelation of the flour-water solubles was first described by Durham (1925) and later shown to be caused by the pentosans (Baker et

al 1943). A critical pentosan concentration must be reached before gelation occurs (Baker et al 1943). Generally, an aqueous extract having a high relative viscosity has a greater capacity to gel.

A number of common oxidants can be used to initiate the gelation. Addition of hydrogen peroxide in the presence of peroxidase, an enzyme native to flour, has been studied most extensively. Twenty to 40 ppm hydrogen peroxide is generally reported as the optimal oxidant level (Baker et al 1943). If excess oxidant is used either no gel forms or a gel forms but quickly dissolves (Baker et al 1943, Painter and Neukom 1968). The rate of gelation increased as pH was decreased. The reaction is instantaneous at pH 4 while at high pH's no gel forms (Painter and Neukom 1968).

Interference with gel formation

Certain organic compounds, such as phenols, ascorbic acid, and aryl and alkyl amines, have been reported to interfere with gel formation (Neukom et al 1967). Baker et al (1943) reported that both flour extracts and purified pentosans from sprouted wheat flours did not gel. High ash flours also reportedly will not gel (Neukom and Markwalder 1978).

Variation in the initial viscosity of water-solubles results from solubility differences (Markwalder 1975, Hoseney et al 1969). It has been suggested that enzyme activity was responsible for variations in the initial viscosity of water solubles, the gradual decrease in the viscosity of water solubles with time, and liquification of purified gels (Durham 1925, Baker et al 1943, Neukom and Markwalder 1978, Martinez 1985). Elevated temperatures purportedly affect polymer

solubility by increasing enzymatic degradation of water-insoluble pentosans to a water-soluble form via transarabinosylation (Preece and Hobkirk 1955, D'Appolonia and Kim 1976). Decreases in the viscosity of aqueous dispersions as well as the liquification of gels may result from naturally occurring enzymes believed to attack internal glycosidic linkages in the pentosan molecule (Preece and MacDougall 1958). Regions of approximately 5 unbranched xylose units are suspected to be susceptible to such enzymatic attack. To avoid enzymatic changes aqueous extracts are heated or the flour is extracted with hot 70 to 80% ethanol (Baker et al 1943, Pence et al 1950, Montgomery and Smith 1956, Preece and MacDougall 1958, Simpson 1954).

Current evidence suggests that the enzyme systems required for pentosan degradation are not present at substantial levels in wheat. Wheat flour is deficient in endoxylanase (Preece and MacDougall 1958), the enzyme responsible for the rapid decrease in viscosity. Frequently cited studies on the enzymolysis of wheat pentosans have utilized exogenous enzyme sources (Kulp 1967, Simpson 1954, Wrench 1965, Mauritzen and Stewart 1965).

When gelation is promoted by addition of hydrogen peroxide, substantial amounts of carbohydrate chains are split in the process, ending up as soluble material (Painter and Neukom 1968). Smidsrod et al (1965) attributed viscosity decreases, of alginic acid, to random oxidative splitting of the carbohydrate chains by hydroxyl radicals produced from the hydrogen peroxide and catalytic amounts of adventitious ferric ions.

Oxidative gelation

Numerous hypotheses concerning the mechanism of oxidative gelation have been developed. None have been substantiated. Ferulic acid associated with the polysaccharides may be involved in crosslinking pentosans (Geissmann and Neukom 1973, Fausch et al 1963, Neukom 1976, Hoseney and Faubion 1981). The ultraviolet spectrum of water-solubles shows absorption maxima at 320 nm (ferulic acid) and 280 nm (aromatic absorption) (Kuendig et al 1961). Ferulic acid is assumed to be involved in gelation because the absorption at 320 nm decreases during the oxidative gelation.

Detection of diferulic acid by thin layer chromatography (TLC) in oxidized crude pentosan solutions, after saponification, prompted Geissmann and Neukom (1973) to suggest that cross-linking occurred through oxidative phenolic coupling of adjacent ferulic acid residues (Fig. 2a).

There are conflicting reports regarding the involvement of protein in gel formation. Aqueous flour extracts treated with proteolytic enzymes lose their capacity to gel and gels treated with proteolytic enzymes liquefy (Painter and Neukom 1968, Baker et al 1943). A strong interaction between pentosans and proteins occurs in the presence of oxidizing agents (Udy 1957). Gel formation of protein free aqueous extracts have been reported (Morita et al 1974). This suggests gelation can occur with or without the participation of proteins.

Neukom and Markwalder (1978) suggest two ways in which protein may be involved in gelation. First, they propose that ferulic acid

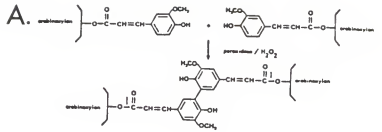
Figure 2. Proposed mechanisms of oxidative gelation.

A. Oxidative phenolic coupling of ferulic acid residues.

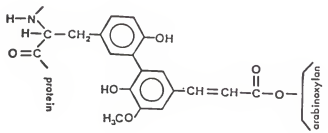
B. Cross-linking of ferulic acid residue with tyrosine.

C. Cross-linking via protein thiyl radical added to activated double bond of ferulic acid residue.

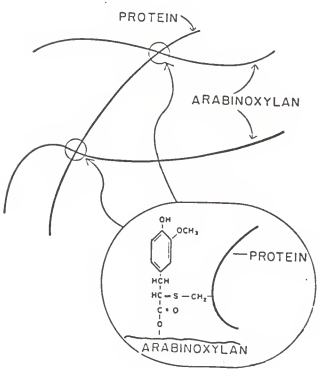
A.



B.


protein
arabinoxylan

C.


PROTEIN
ARABINOXYLAN
PROTEIN
ARABINOXYLAN

could be linked to a N-terminal amino group forming a pseudo-peptide linkage. Secondly, they suggest that the ferulic acid associated with the pentosan could cross-link with the amino acid tyrosine (Fig. 2b). A conflicting hypothesis is that a protein thiyl radical adds to the activated double bond of ferulic acid (Hoseney and Faubion 1981, Fig. 2c).

Functional properties

Although water-soluble pentosans represent only 0.5 to 0.8% of the wheat endosperm, they do influence the physical and functional properties of dough. Pentosans act primarily as water regulators because of their high water-binding capacity. Presence of pentosans increases water absorption capacity of flour (Durham 1925) and affects the viscous behavior of dough (Dreese et al 1988).

Removal of the water-soluble fraction eliminates dough breakdown during mixing (Schroeder and Hoseney 1978, Kerr 1987). It is believed that activated double bond compounds associated with pentosans contribute to dough breakdown. Pentosans have also been reported to influence the staling of bread (Kim and D'Appolonia 1977). Their ability to retain large amounts of water causes a decrease in the firming rate but increases the rate of starch retrogradation (Rogers et al 1988).

Another important functional property of pentosans is their ability to retain gas in doughs (Hoseney 1984). Removal of the water-soluble fraction reduces loaf volume (Hoseney et al 1969). It has been speculated that the oxidative gelation of water-soluble pentosans may be related to oxidative improvement of dough (Hoseney 1984).

MATERIALS AND METHODS

Materials

Flour. Commercial straight grade malted flour containing 13.0% protein, 13.4% moisture, and 0.47% ash was used (Ross Mills, Wichita, KS). The malted flour gave higher viscosity readings in preliminary testing and was used in subsequent studies.

Enzymes. Type I horseradish peroxidase (94 purpurogallin units per mg) was from Sigma Chemical Company, St. Louis, MO.

Chemicals. Unless specified, all chemicals used were reagent grade. Ferulic acid (4-hydroxy-3-methoxycinnamic acid) was from ICN Pharmaceuticals, Inc., Plainview, NY; L-cysteine HCl from Nutritional Biochemicals Corp, Cleveland, OH; 3% H_2O_2, ethanol (95%), ethyl acetate, fumaric acid, phenol, xylose, and sodium hydroxide, were obtained from Fisher Scientific Co., Fair Lawn, NJ. Sulfuric acid, hydrochloric acid, and vanillic (4-hydroxy-3-methoxybenzoic) acid were from Sigma Chemical Co (St. Louis, MO).

Methods

Viscosity measurements. Viscometric techniques were used to measure the relative viscosity of aqueous flour extracts. Cannon-Fenske (size 50) capillary viscometers, held in a constant temperature water bath ($30 \pm 0.1\ ^{o}C$), were calibrated with distilled water. Each measurement consisted of pipetting 5 ml solution into the tube and allowing a 5 min temperature equilibration period. The time required

for a constant volume of solution to flow through the capillary, pt. A to pt. B, (Fig. 3) was recorded and relative viscosity was calculated as:

$$\text{Relative viscosity} = \frac{\text{flow time of the aqueous extract}}{\text{flow time of distilled water}}$$

Preparation of flour extracts. Preparation of the aqueous flour extracts is shown schematically in Fig. 4. Flour and water were combined at a ratio of 1:5 (w/v). The resultant slurries were stirred for specific time intervals (0, 30, 60, or 90 min) and then centrifuged 15 min (360 x G). The supernatant was decanted and the precipitate discarded. The supernatant was recentrifuged 10 min (1000 x G). The resultant aqueous extracts were used without further purification.

The time flour and water remained in contact prior to centrifugation is referred to as time before centrifugation (TBC). A no-time extraction (TBC=0) refers to centrifugation immediately after uniform slurry preparation. Samples having TBC>0 were stirred every 5 min until centrifugation.

pH optima determination. After centrifugation the pH of aqueous flour extracts (1:5) was adjusted with either 0.1 N HCl or 0.1 N NaOH. The pH range evaluated was pH 2.0 to 8.5 in increments of 0.5. Relative viscosity of aqueous extracts was measured at 0, 2, 4 or 6 hr after centrifugation (TAC). To obtain good reproducibility, it is critical to control the time that measurements are made.

Figure 3. A Cannon-Fenske capillary tube. Solution flow is timed between pt. A to pt. B.

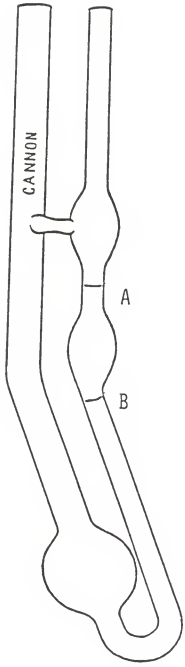
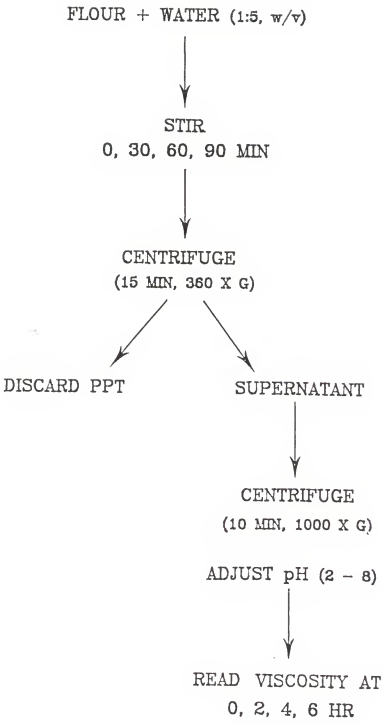

CANNON
A
B

Figure 4. Flow diagram for isolation of aqueous extracts used in determination of pH optima.


FLOUR + WATER (1:5, w/v)
STIR
0, 30, 60, 90 MIN
CENTRIFUGE
(15 MIN, 360 X G)
DISCARD PPT
SUPERNATANT
CENTRIFUGE
(10 MIN, 1000 X G)
ADJUST pH (2 – 8)
READ VISCOSITY AT
0, 2, 4, 6 HR

Statistical Analysis. SAS, a statistical analysis system, was used to analyze the relative viscosity of aqueous extracts at all TBC and TAC combinations. A backwards, step-wise elimination procedure provided a quadratic viscosity prediction model for the system with R^2 = 0.90 and α = 0.1. Regression analyses were performed to ensure the model gave a normalized distribution.

Carbohydrate quantitation. Total carbohydrate content of 1:5 aqueous extracts were estimated using a modified phenol sulfuric procedure (Southgate, 1976). A stock solution of 70 μg D-xylose per ml was used as a standard. The supernatant was diluted 1:500 prior to analysis. Absorbance was measured at 480 nm.

Optimum oxidant requirement. Aqueous extracts, prepared at four flour:water ratios (1:10, 1:5, 1:3, and 1:2), were treated with increasing levels of H_2O_2. Isolation of aqueous extracts as shown in Fig. 4, except the supernatant pH was not adjusted. Aqueous extracts were treated with oxidant for 15 min at room temperature. The optimum oxidant level, in ppm, was based on flour weight.

Oxidation of aqueous extracts. Flour and water were combined in ratios of 1:10, 1:5, 1:3, and 1:2 (w/v). A no-time extraction was used (centrifugation 5 min after water addition). Aqueous extracts were treated with 15 ppm hydrogen peroxide (H_2O_2) for 15 min at room temperature. Increases in relative viscosity were determined for all flour:water ratios. Analysis of variance was performed and least significant differences calculated.

Rheologically active compounds. Aqueous solutions of ferulic acid (250 ppm), vanillic acid (250 ppm), and fumaric

acid (250 ppm) were prepared by bringing the solution to pH 7 with 1.0 N NaOH. Cysteine solution (250 ppm) was prepared in distilled water.

The effects of 250 ppm (flour weight basis) of rheologically active compounds were evaluated at flour:water ratios of 1:10, 1:5, 1:3, and 1:2 (Fig. 5). A no-time extraction was used. The rheologically active compounds were added immediately and H_2O_2 (15 ppm) was added after 5 min. The relative viscosity was recorded.

Cysteine. Aqueous extracts, prepared at flour:water ratios of 1:3 and 1:2 (w/v) were treated with increasing concentrations of H_2O_2 at a constant level of cysteine (312 ppm and 208 ppm respectively). Relative viscosity of the aqueous extracts was determined.

Flour extraction.

Ethanol (80%) extraction. Flour was extracted with 80% ethanol 1:3 (w/v) following the procedure of Martinez (1985, Fig. 6). Flour and ethanol were stirred 30 min, and then filtered through a Buchner funnel (Whatman #4). The extraction was repeated twice more. The resulting flour was dried overnight and ground to a uniform particle size in a coffee-grinder.

Peroxidase (190 purpurogallin units, PU) was added to extracts from chemically treated flours to ensure peroxidase activity.

Figure 5. Schematic for addition of rheologically active compounds to aqueous extracts.

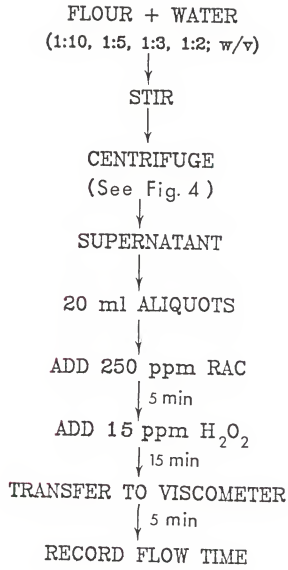
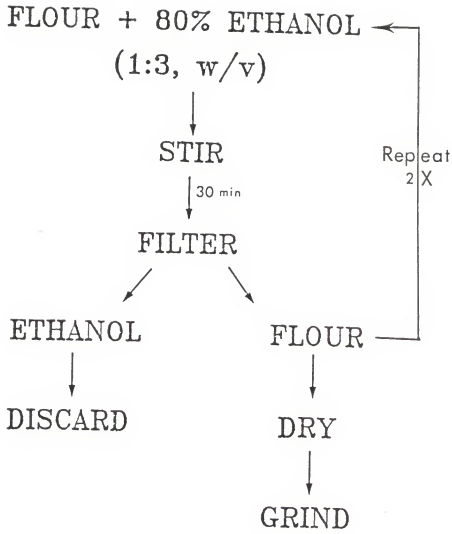

FLOUR + WATER
(1:10, 1:5, 1:3, 1:2; w/v)
STIR
CENTRIFUGE
(See Fig. 4)
SUPERNATANT
20 ml ALIQUOTS
ADD 250 ppm RAC
5 min
ADD 15 ppm H_2O_2
15 min
TRANSFER TO VISCOMETER
5 min
RECORD FLOW TIME

Figure 6. Flow diagram for extraction of flour with 80% ethanol.


FLOUR + 80% ETHANOL
(1:3, w/v)
STIR
30 min
FILTER
ETHANOL
DISCARD
FLOUR
DRY
GRIND
Repeat
2X

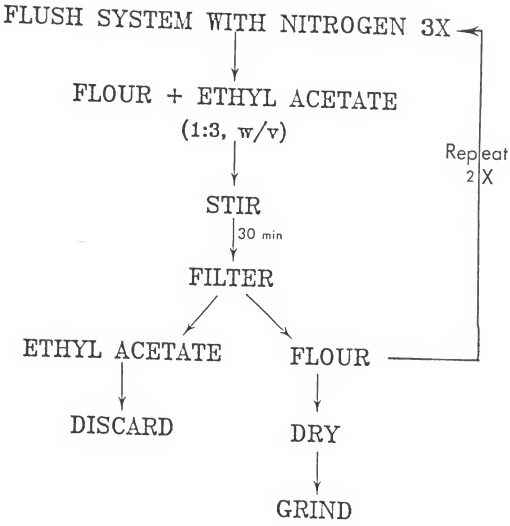
Ethanol (95%) extraction. Substantial amounts of gliadin were removed by Martinez's (1985) extraction procedure. To determine if ethanol extraction removed an interfering compound, flour was extracted with 95% EtOH (1:3, w/v) as shown in Fig. 6. A single extraction was performed. The ethanol extract was collected and air dried.

If ethanol extraction removed an interfering substance from flour, then addition of the ethanol extract to extracted flour, at the original level should stop gelation. Aqueous extracts were prepared at flour:water ratios of 1:3 and 1:2.

It could be possible that ethanol did not remove an active compound but altered the pentosan fraction. To test this, flour and 95% ethanol were stirred 30 min, poured into a pan, and dried overnight without separation. The dried flour was ground with a coffee-grinder. Aqueous extracts were prepared from these flours at ratios of 1:3 and 1:2 (flour:water, w/v). Extracts were treated with 190 PU peroxidase and 15 ppm H_2O_2. The relative viscosity of aqueous extracts was determined.

Ethyl acetate extraction. Flour was extracted with ethyl acetate (1:3, w/v), under an inert (N_2) atmosphere (Fig. 7). Aqueous extracts were treated with 190 PU peroxidase and 15 ppm H_2O_2. Relative viscosity of aqueous extracts was determined.

Figure 7. Flow diagram for extraction of flour with ethyl acetate.


FLUSH SYSTEM WITH NITROGEN 3X
FLOUR + ETHYL ACETATE
(1:3, w/v)
STIR
30 min
FILTER
ETHYL ACETATE
FLOUR
DISCARD
DRY
GRIND
Repeat
2 X

EtOH and EtOAc flour extraction. Flour was first extracted with 80% EtOH as previously described in Fig. 6. And then the flour was extracted with EtOAc as described in Fig. 7. This provided a flour that presumably contained neither free sulfhydryl groups nor free activated double bond compounds.

RESULTS AND DISCUSSION

Determination of the pH optimum. The relative viscosity of aqueous extracts increased as the time water and flour remain in contact increased and decreased as a function of the time the extract was held prior to measurement (Martinez 1985). Based on the literature (Preece and Hobkirk 1955) we presumed that an enzyme was acting on the insoluble material and making it soluble. We also presumed that once the insoluble substrate was removed by centrifugation, that enzyme proceeded to degrade the soluble material. If an enzyme is responsible for changes in the physical properties of aqueous extracts then it should be possible to determine the pH optimum for that enzyme.

Effect of time before centrifugation. The relative viscosity of aqueous extracts increased as the time increased that flour and water remained in contact prior to centrifugation (Fig. 8). The concentration of carbohydrates present in the extracts also increased with time before centrifugation (Table I). The increase in viscosity was curvilinear. This illustrates the characteristic direct, nonlinear relationship between concentration of a solute and viscosity at a constant temperature (Charm 1981). Figure 8 also suggests that, with time, flour components, presumably the water-insoluble pentosans, were becoming soluble.

Effect of time after centrifugation. The relative viscosities of aqueous extracts decreased as time after centrifugation increased

Figure 8. Increase in relative viscosity with increasing time that flour and water remain in contact prior to centrifugation. Time after centrifugation was zero.

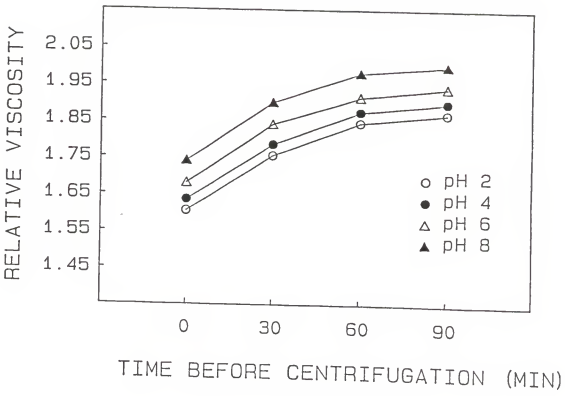

RELATIVE VISCOSITY
2.05
1.95
1.85
1.75
1.65
1.55
1.45
0
30
60
90
TIME BEFORE CENTRIFUGATION (MIN)
pH 2
pH 4
pH 6
pH 8

Table I. Effect of increasing time before centrifugation on the concentration of aqueous extracts

Time Before Centrifugation (min)	mg xylose equivalents/ml
0	7.5
30	11.5
60	14.1
90	19.4

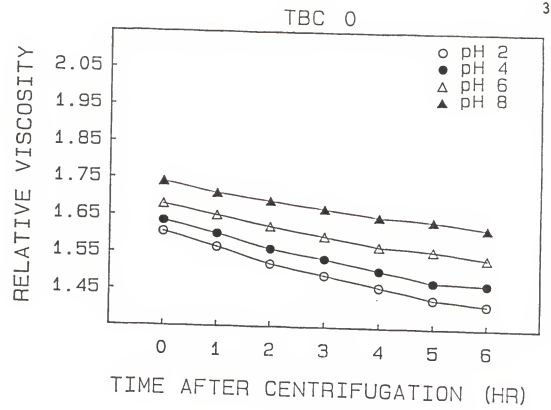
a decrease in molecular weight or concentration. Adjustment of the pH of aqueous extracts directly affected the initial viscosity (Fig. 8). The higher pHs gave higher relative viscosities.

The pH optima for pentosanases has been reported as pH 4.5-5.0 (Preece and MacDougall 1958). Thus, we would expect to see the most rapid decrease in viscosity at that pH range. The decrease in relative viscosity, for all pHs evaluated (pH 2 to pH 8.5), gave curves which paralleled each other. Only four curves are shown in Figures 9 and 10 to avoid confusion. The relative viscosity of aqueous extracts at pH 4.0, 4.5 and 5.0 did not decrease more rapidly than that found with the other pHs tested. This suggests that the decrease in relative viscosity of aqueous extracts was not the result of enzymatic activity.

However, the decrease in viscosity was pH dependent. The relative viscosity of aqueous extracts at low pH values continued to decrease as time after centrifugation increased. At high pH, there was a plateau in the decrease of relative viscosity following 4 hr after centrifugation. The pronounced decrease in viscosity at pH 2 may be attributed to the acid lability of the hemiacetal bond. Cleavage of the arabinose side chains may allow the xylan chain to associate and precipitate from solution, resulting in a decreased viscosity.

The absence of a pH optimum for the decrease in viscosity, suggested that an enzyme was not responsible for either the increase in viscosity with time or the decrease in viscosity with time.

Figure 9. Effect of pH at 0 and 30 min after centrifugation on the relative viscosity of aqueous extracts.


TBC 0
pH 2
pH 4
pH 6
pH 8
RELATIVE VISCOSITY
2.05
1.95
1.85
1.75
1.65
1.55
1.45
0
1
2
3
4
5
6
TIME AFTER CENTRIFUGATION (HR)

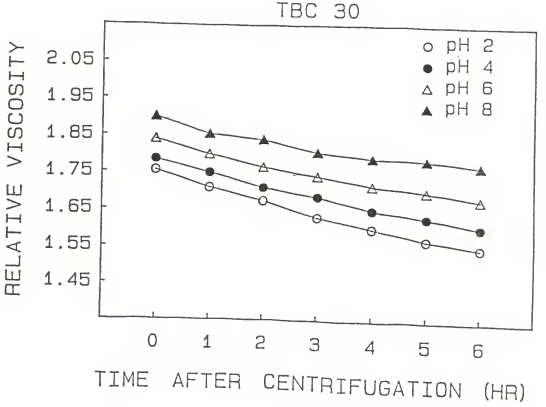
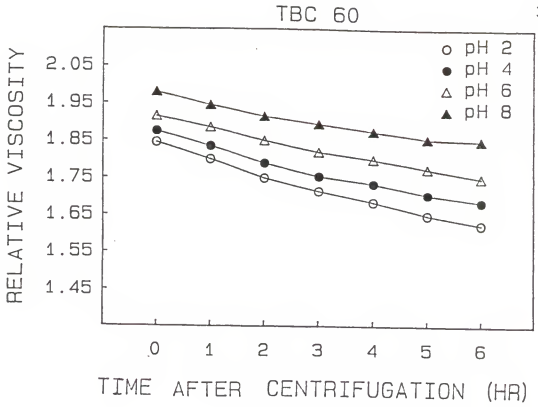
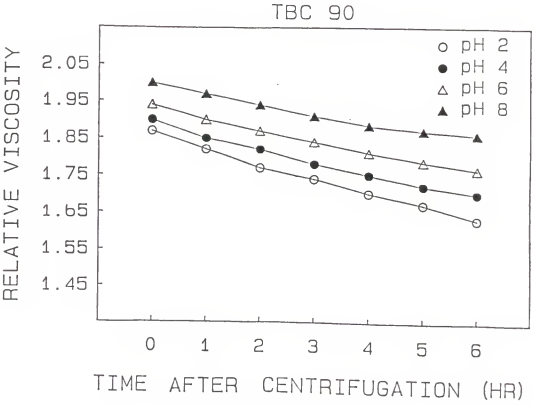

TBC 30
pH 2
pH 4
pH 6
pH 8
RELATIVE VISCOSITY
2.05
1.95
1.85
1.75
1.65
1.55
1.45
0
1
2
3
4
5
6
TIME AFTER CENTRIFUGATION (HR)

Figure 10. Effect of pH at 60 and 90 min after centrifugation on the relative viscosity of aqueous extracts.


TBC 60
RELATIVE VISCOSITY
2.05
1.95
1.85
1.75
1.65
1.55
1.45
pH 2
pH 4
pH 6
pH 8
0
1
2
3
4
5
6
TIME AFTER CENTRIFUGATION (HR)


TBC 90
RELATIVE VISCOSITY
2.05
1.95
1.85
1.75
1.65
1.55
1.45
pH 2
pH 4
pH 6
pH 8
0
1
2
3
4
5
6
TIME AFTER CENTRIFUGATION (HR)

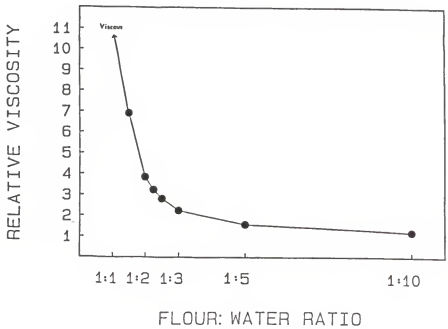
This finding is consistent with reports that wheat is deficient in enzymes needed to degrade pentosans (Preece and Hobkirk 1955).

Oxidative gelation. Preliminary attempts to reproduce the findings reported by Hoseney and Faubion (1981) were unsuccessful. Thus, the water-soluble system was reevaluated to identify factors contributing to oxidative gelation.

Baker et al (1943) showed that viscosity increased as the flour:water ratio increased. The effect of an increased flour:water ratio on the relative viscosity of flour extracts is shown in Fig. 11. At a 1:1.5 flour:water ratio, the relative viscosity of the flour extract (6.39) is 5 times greater than the relative viscosity of a 1:10 flour:water extract (1.25). Increased relative viscosity reflects concentration of water-soluble polymers in the aqueous extract.

Effect of oxidant level and flour:water ratio on gelation. Hoseney and Faubion (1981) showed that addition of an oxidant to aqueous extracts (1:10, flour:water) caused a 10% increase in flow time. Evaluation of several flours, following Hoseney and Faubion's (1981) procedure, did not result in the formation of a gel at a 1:10 flour:water ratio. Inability to form a gel may be because a critical pentosan concentration must be reached before gelation can occur (Baker et al 1943, Neukom and Markwalder 1978, Martinez 1985). On the other hand, inability to form a gel may have been because of an inappropriate oxidant concentration. The oxidant level reportedly used was 1.25 ml of 30% H_2O_2 per 100 ml water solubles

Figure 11. Effect of flour:water ratio on the relative viscosity of aqueous extracts.


RELATIVE VISCOSITY
11
10
9
8
7
6
5
4
3
2
1
Viscous
1:1
1:2
1:3
1:5
1:10
FLOUR: WATER RATIO

(15,000 ppm based on a flour weight of 25g). That oxidant level produced excessive bubbles. Those bubbles could not be removed by adding catalase and degassing the solution, as outlined in their procedure. Approximately 45 min were required to "briefly" degas the solution. This raised a question of whether the increased flow time reflected an increase in viscosity or the presence of bubbles in the capillary neck.

Optimum oxidant requirement. If addition of 80 ppm H_2O_2 to aqueous extracts inhibited gelation (Baker et al 1943), then perhaps inability to form a gel following Hoseney and Faubion's procedure (1981) was because of an excessive oxidant level. Evaluation of the oxidant level at a 1:2 flour:water ratio showed that the increase in relative viscosity passed through a maximum as the oxidant level increased (Fig. 12). The relative viscosity of aqueous extracts treated with 15 to 40 ppm H_2O_2 (based on flour weight) were not significantly different (Table II). Addition of higher levels of oxidant caused a decrease in viscosity. Using low levels of H_2O_2 eliminated the need to add catalase and degas the solution.

Oxidation of aqueous extracts. The relative viscosity of aqueous extracts increased as the flour to water ratio decreased (Baker et al 1943, Martinez 1985). Because gelation requires a critical pentosan concentration, it seemed reasonable that inability to produce a gel was because of the low flour:water ratio of 1:10. Increases in viscosity, at all four flour:water ratios, were examined at an oxidant level of 15 ppm (based on flour weight). The relative viscosity between each flour:water ratio were significantly

Figure 12. Effect of oxidant level on the relative viscosity of aqueous extracts prepared at a 1:2 flour:water ratio.

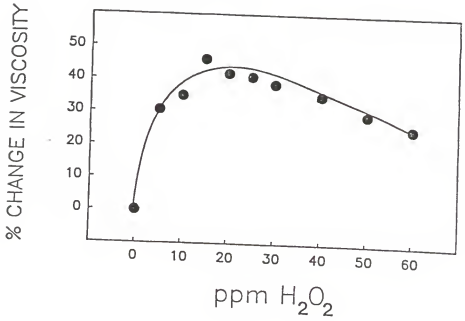

% CHANGE IN VISCOSITY
50
40
30
20
10
0
0
10
20
30
40
50
60
ppm H_2O_2

Table II. Effect of Oxidant Levels at each Flour:Water Ratio*

	FLOUR:WATER RATIO			
ppm H_2O_2	1:10	1:5	1:3	1:2
0	1.25^{A}	1.63^{B}	2.38^{C}	4.47^{G}
5	1.63^{A}	1.72^{B}	2.70^{E}	5.84^{H}
10	1.27^{A}	1.72^{B}	2.76^{F}	6.30^{I}
15	1.27^{A}	1.75^{B}	2.78^{F}	6.53^{J}
20	1.27^{A}	1.75^{B}	2.75^{F}	6.34^{I}
25	1.26^{A}	1.73^{B}	2.75^{F}	6.30^{I}
30	1.26^{A}	1.69^{B}	2.74^{F}	6.20^{I}
40	1.24^{A}	1.67^{B}	2.71^{F}	6.05^{I}
50	1.24^{A}	1.67^{B}	2.68^{D}	5.79^{H}
60	1.23^{A}	1.64^{B}	2.52^{F}	5.61^{H}

* Means with different letters are significantly different, both within and between columns. LSD=0.07, MSE=0.01.

different (Table III). In dilute systems (1:10 and 1:5), the addition of oxidant has a negligible effect, while in concentrated systems a gel forms with oxidant addition. Malted Ross flour, at a 1:1.5 flour:water ratio, formed a gel. A strong viscoelastic gel formed at a flour:water ratio of 1:1.

Effect of rheologically active compounds. Hoseney and Faubion (1981) showed that the addition of certain rheologically active compounds (RACs) stopped increases in viscosity with oxidation. They reasoned that if the rheologically active compound was involved in the reaction, then addition of the rheologically active compound should stop increases in viscosity. Martinez (1985) was unable to reproduce their findings.

Ferulic acid is esterified to the largest molecular weight arabinoxylan fraction in aqueous extracts of flour (Yeh et al 1980). UV studies showed the ferulic acid peak at 320 nm disappeared after gel formation (Kuendig et al 1961). Thus, esterified ferulic acid is presumably involved in the oxidative gelation of the water-soluble pentosans (Neukom et al 1967). If esterified ferulic acid is involved in gelation, then addition of free ferulic acid to water-solubles should inhibit increases in viscosity by reacting before the less mobile esterified form.

Addition of 250 ppm ferulic acid to the more concentrated extracts (from flour:water ratios of 1:3 and 1:2), stopped increases in viscosity. At flour:water ratios of 1:10 and 1:5, differences in viscosity were not significant and will be ignored in subsequent studies.

Table III. Effect of the Flour:Water Ratio and Oxidation on the Relative Viscosity of Aqueous Flour Extracts*

RATIO	CONTROL	H_2O_2
1:10	1.25^{A}	1.27^{A}
1:5	1.65^{B}	1.75^{B}
1:3	2.44^{C}	2.86^{D}
1:2.5	2.92^{E}	3.53^{F}
1:2.25	3.95^{G}	4.26^{H}
1:2	4.33^{I}	6.25^{J}
1:1.5	6.39^{K}	10.84^{L}
1:1	40.56^{M}	GEL

* Means with different letters are significantly different, both within and between columns. LSD=0.09, MSE=0.02.

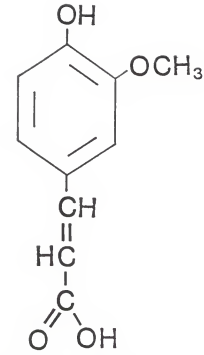
How is ferulic acid involved in oxidative gelation? Ferulic acid (Fig. 13) has three potential reactive sites: two on the aromatic ring and one at the activated double bond. These reactive sites could serve to cross-link polymers and increase the molecular size of the polymers. Neukom and Markwalder (1978) suggested involvement of the aromatic nucleus to form diferulic acid bridges. Sidhu et al (1980) and Hoseney and Faubion (1981) point to the activated double bond as the cross-linking site. Hoseney and Faubion (1981) suggested a protein thiyl radical added to the activated double bond.

To determine whether cross-linking occurs through the aromatic ring or activated double bond, structural analogs of ferulic acid (Fig. 13) were added to aqueous extracts prior to oxidation. Vanillic acid contains an aromatic ring but no activated double bond. Fumaric acid possesses an activated double bond but not an aromatic nucleus.

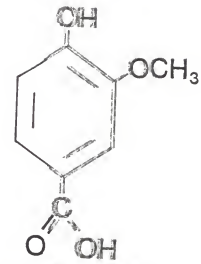
Addition of 250 ppm vanillic acid stopped increases in viscosity at 1:3 and 1:2 flour:water ratios (Table IV). The increase in viscosity with 250 ppm fumaric acid present was equivalent to the H_2O_2 treated sample. The ability of vanillic acid to stop increases in viscosity points to the aromatic nucleus as the active center. These results contradict with those reported by Hoseney and Faubion (1981) but agree with those of Martinez (1985).

Cysteine. Hoseney and Faubion (1981) suggested that cysteine was added to the activated double bond during the gelation reaction. Sidhu et al (1980b) showed that ^{14}Cys added to water-soluble pentosans under UV light and presumably reacted with ferulic acid. To determine if cysteine participated in the reaction, 250 ppm cysteine was added

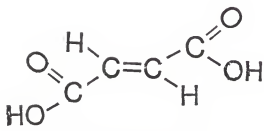
Figure 13. Structures of ferulic acid, fumaric acid, vanillic acid, and cysteine.



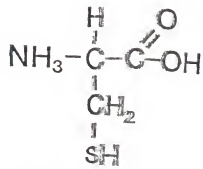
Ferulic Acid



Vanillic Acid



Fumaric Acid



Cysteine

Table IV. Effect of Rheologically Active Compounds on the Relative Viscosity of Water-solubles*

RATIO	CONTROL	H_2O_2	FERULIC	FUMARIC	VANILLIC	CYSTEINE
1:10	1.24^{A}	1.26^{A}	1.24^{A}	1.26^{A}	1.24^{A}	1.21^{A}
1:5	1.58^{B}	1.71^{B}	1.62^{B}	1.69^{B}	1.56^{B}	1.62^{B}
1:3	2.26^{C}	2.59^{D}	2.31^{C}	2.71^{D}	2.35^{C}	2.27^{C}
1:2	3.84^{E}	5.46^{G}	4.12^{F}	5.32^{G}	4.27^{F}	3.95^{E}

* Means with different letters are significantly different, both within and between rows. LSD = 0.17, MSE = 0.04.

to aqueous extracts. Addition of 250 ppm cysteine stopped gelation (Table IV).

A concern raised at this point was whether the cysteine was reacting with the ferulic acid or reacting with the H_2O_2 and, thus, inhibiting gelation by consuming the oxidant. If cysteine stopped the increase in viscosity by reacting with the H_2O_2, then addition of excess H_2O_2 should result in increased viscosity. Addition of cysteine interfered with increases in viscosity at all H_2O_2 levels (Table V). The addition of excess H_2O_2 does not give an increase in viscosity (Fig. 14). This suggests that cysteine may be interfering with gelation by reacting with the active center of the polymers and preventing the necessary cross-linking.

Effect of **flour extraction.** Martinez (1985) found that extracting flour with 80% ethanol (EtOH) shifted the critical concentration required for gelation. She concluded that this shift was because some unidentified substance(s), which interfered with gelation, had been removed. Free sulfhydryl containing compounds were implicated as the interfering substance(s).

Flour water-solubles, prepared from flour extracted with 80% ethanol, formed a gel at a flour:water ratio of 1:2 when treated with 15 ppm H_2O_2 (Table VI). The ability of the aqueous extract to gel in a less concentrated system suggests that extraction of flour with ethanol removed some inhibitory substance.

If free ferulic acid were naturally present in aqueous extracts, then this may be why such a concentrated system is required for gelation. Flour was extracted with ethyl acetate (EtOAc) to remove

Table V. Effect of increasing levels of H_2O_2 at a constant cysteine concentration*

1:3 FLOUR:WATER RATIO

equivalence at 33.8 pppm H_2O_2 per 312 ppm Cys

H_2O_2	CYS	RELATIVE VISCOSITY
0	0	2.17 ± 0.11^A
10	0	2.52 ± 0.07^B
10	312	2.25 ± 0.04^A
0	0	2.24 ± 0.04^A
30	0	2.50 ± 0.06^B
30	312	2.30 ± 0.04^A
0	0	2.17 ± 0.03^A
52	0	2.53 ± 0.03^B
52	312	2.28 ± 0.01^A
0	0	2.17 ± 0.02^A
74	0	2.49 ± 0.04^B
74	312	2.27 ± 0.01^A

1:2 FLOUR:WATER RATIO

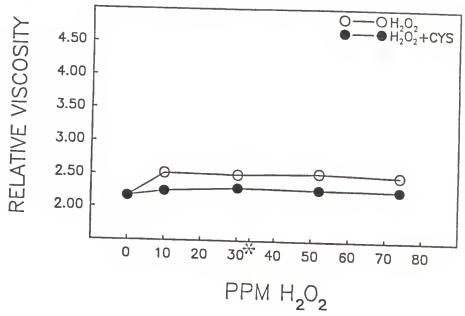
equivalence at 22.5 ppm H_2O_2 per ppm Cys

H_2O_2	CYS	RELATIVE VISCOSITY
0	0	3.95 ± 0.15^C
7	0	5.64 ± 0.14^D
7	208	4.23 ± 0.20^C
0	0	3.83 ± 0.19^C
20	0	5.75 ± 0.31^D
20	208	4.21 ± 0.25^C
0	0	3.63 ± 0.34^C
34	0	5.50 ± 0.22^D
34	208	4.03 ± 0.36^C
0	0	3.72 ± 0.10^C
49	0	5.34 ± 0.08^D
49	208	4.09 ± 0.19^C

* Means with different letters are significantly different. LSD=0.39, MSE=0.05.

Figure 14. Effect of adding increasing levels of hydrogen peroxide (H_2O_2), at a constant cysteine concentration, on increases in viscosity at flour:water ratios of 1:3 (A) and 1:2 (B). * indicates the point of chemical equivalence for H_2O_2 and cysteine.

A.


RELATIVE VISCOSITY
4.50
4.00
3.50
3.00
2.50
2.00
0
10
20
30
40
50
60
70
80
PPM H_2O_2
H_2O_2
H_2O_2+CYS

B.

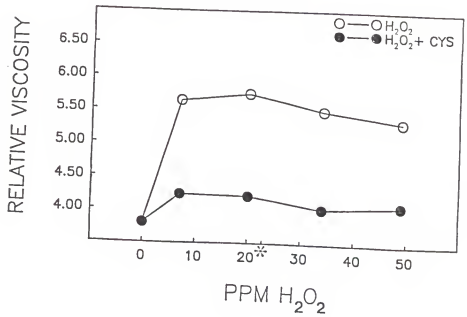

RELATIVE VISCOSITY
6.50
6.00
5.50
5.00
4.50
4.00
0
10
20
30
40
50
PPM H_2O_2
H_2O_2
H_2O_2+ CYS

Table VI. Effect of Extracting Flour with EtOH, EtOAc, or a Combination of EtOH and EtOAc on Relative Viscosity*

	TREATMENT		
FLOUR:WATER	EtOH	EtOAc	EtOH & EtOAc
1:10			
Control	1.24^{A}	1.24^{A}	1.24^{A}
H_2O_2	1.32^{A}	1.25^{A}	1.25^{A}
1:5			
Control	1.59^{B}	1.61^{B}	1.60^{B}
H_2O_2	1.70^{B}	1.71^{B}	1.78^{B}
1:3			
Control	2.32^{C}	2.32^{C}	2.51^{C}
H_2O_2	3.13^{E}	2.64^{D}	3.37^{E}
1:2			
Control	4.47^{F}	4.40^{F}	4.59^{F}
H_2O_2	Gel	6.24^{G}	Gel

* Means with different letters are significantly different, both within and between rows. LSD=0.40, MSE=0.14.

any free activated double bond compounds (Jackson 1983). EtOAc extraction of flour did not promote an increase in viscosity (Table VI). Flour extracted with both 80% EtOH and EtOAc did not show an additional increase in viscosity as a result of extraction with EtOAc. Only EtOH extraction achieved removal of the presumed inhibitory substance.

Extraction of flour with 80% ethanol also removes the majority of the gliadin protein fraction. To determine whether removal of the gliadin protein fraction was responsible for the decreased concentration requirement of gelation, flour was extracted with 95% EtOH.

Extraction of flour with 95% EtOH caused aqueous extracts, prepared at a 1:2 flour:water ratio, to gel. Subjecting the flour to EtOH did not cause the extracts to gel (Table VII). Therefore, the gelling ability of the 95% EtOH extracted flour was not a result of exposure to ethanol. Addition of the EtOH extract back to EtOH extracted flour, at the same level of extraction, inhibited the formation of a gel at a flour:water ratio of 1:2 (Table VII). It appears that addition of the ethanol extract to the aqueous extracts reintroduces some interfering material. The nature of this interfering material has not been determined. There were no significant differences between ethanol treatments at a flour:water ratio of 1:3 (Table VII).

Table VII. Effect of Different Ethanol Treatments on Relative Viscosity*

	FLOUR:WATER RATIO			
	1:2		1:3	
Treatment	Control	H_2O_2	Control	H_2O_2
Extracted	5.09^A	9.56^C	2.48^D	2.98^E
Stirred	5.11^A	7.49^B	2.50^D	2.90^E
Added extract	5.29^A	7.80^B	2.50^D	3.02^E

* Means with different letters are significantly different, both within and between rows. LSD=0.41, MSE=0.22.

SUMMARY

This study has clarified some of the contradicting information about the gelation of wheat flour water-solubles. Gelation of aqueous flour extracts requires a concentrated system. Oxidation of dilute aqueous flour extracts did not show a significant increase in viscosity. Extraction of flour with EtOH decreases the concentration requirement for gelation by apparently removing some interfering substance. The oxidant level used to promote gelation is a critical factor. Excess oxidant appears to degrade the pentosans and, thus, causes a decrease in viscosity. Changes in the physical properties of aqueous flour extracts do not appear to be a result of enzymatic degradation.

The mechanism of oxidative gelation of wheat flour water-solubles remains confusing. However, the aromatic ring of ferulic acid appears to serve as the cross-linking center for the pentosan polymers. Cysteine may also participate in this reaction. Now that the system is better understood, determination of the mechanism of oxidative gelation should be explored.

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ACKNOWLEDGEMENTS

Sincere gratitude is extended to Dr. Carl Hoseney for his endless questions and answers, patience, and guidance throughout the course of this project; even when I wanted to strangle him in frustration. Assistance of my committee members, Dr. Carol Klopfenstein and Dr. Jon Faubion is appreciated.

A warm thanks to my parents for teaching me to believe in myself and for providing their support and understanding. A special thanks goes to Mike Lasch for making me laugh amid the chaos and the short-lived turmoil that accompanies this type of endeavor.

FACTORS INVOLVED IN THE OXIDATIVE GELATION OF WATER-SOLUBLE PENTOSANS

by

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B.S., University of Arkansas, 1985

AN ABSTRACT OF A THESIS

submitted in partial fulfillment
of the requirements for the degree

MASTER OF SCIENCE

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1988

Viscometry of wheat flour extracts was used to study factors contributing to oxidative gelation. Addition of hydrogen peroxide to dilute aqueous extracts of wheat flour did not show an increase in viscosity. However, increases in viscosity, with hydrogen peroxide, were observed in more highly concentrated aqueous extracts. Oxidant concentration was a critical factor for gelation. High levels of hydrogen peroxide decreased the increase in viscosity. Addition of ferulic acid, vanillic acid and cysteine stopped increases in viscosity. The mechanism of this oxidative gelation appears to be complicated and influenced greatly by the concentration of the polysaccharide and the oxidant concentration. Flour extracted with 95% ethanol gelled at a lower concentration than the concentration required for nonextracted flour to gel. This suggests that some unknown inhibiting factor is removed by extracting flour with ethanol.