

Partial Purification of a Water-Extractable Rye (*Secale cereale*) Protein Capable of Improving the Quality of Wheat Bread¹

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ABSTRACT

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Rye water-soluble extracts contain a protein fraction that, when added at low concentrations to a straight-dough breadmaking recipe, significantly increased bread volume. Enrichment of the active component is possible by anion-exchange fractionation with diethylaminoethyl-cellulose (DEAE), by ammonium sulfate precipitation, or by using rye bran or shorts milling fractions as the starting material. The active material was not bound to DEAE-cellulose. With ammonium sulfate precipitation, the fractions obtained at 30, 40, and 50% saturation were active in straight-dough baking experiments. Iso-electric focusing revealed that fractions

active in breadmaking invariably contained alkaline protein fractions (pI > 7.5). Inactivation of enzyme material by boiling the water-soluble extract from rye destroyed all breadmaking activity. The activity of the bread improver was additive to that of potassium bromate but not to that of ascorbic acid. It was not counteracted by catalase, showing that it does not work by a mechanism involving the production of hydrogen peroxide. The extract was not able to overcome the detrimental effect on bread quality resulting from mixing dough in a nitrogen atmosphere.

Wheat is a unique cereal in that it can produce good bread. Indeed, when wheat flour is mixed with water, a dough develops because of the properties of the wheat storage (gluten) proteins. The properties of the gluten network are the main determinants of the quality of the resulting bread. The right gluten characteristics lead to a machinable dough with optimum gas retention properties and, finally, to a bread with high volume and a good crumb structure. In addition to the quantity and quality of the gluten proteins, many other flour constituents are important in breadmaking. These include a number of enzymes, lipids, and starch (Hosoney 1994).

Enzymes have theoretical or practical applications in the baking industry. The uses of malt (Dapron and Godon 1987), fungal amylase (Knight 1960), and bacterial amylase (Rubenthaler et al 1965) are well documented; they all supply fermentable sugars to the yeast and degrade damaged starch. Also, use of bacterial amylase improves crumb softness (Dragsdorf and Varriano-Marston 1980). Lipoxigenase, in the form of soybean flour (Sullivan et al 1936), improves the mixing tolerance of dough (Faubion and Hosoney 1981).

The breadmaking performance of strong flour can be improved by addition of proteases (Dapron and Godon 1987). Hemicellulases also have beneficial effects (Ter Haseborg and Himmelstein 1988).

In this article, we describe the isolation and partial purification of a proteinaceous material from rye (*Secale cereale* L.) that is capable of improving the handling properties of wheat flour dough and the volume and crumb structure of the resulting breads (Delcour and Hosoney 1997). Its effect on the bread, in many respects, can be compared to that of an oxidant such as ascorbic acid, and its activity does not require the addition of other substances to the breadmaking recipe.

MATERIALS AND METHODS

Materials

North American wheat flour was a commercial malted flour (Cargill, Wichita, KS). European wheat flour was produced from the wheat cultivar Rector, grown in Belgium, and milled with a Buhler MLU-202 laboratory mill (Uzwil, Switzerland). Two commercial European wheat flours were also used: Uno and Surbi (Ceres, Vilvoorde, Belgium). Approved Methods (AACC 1995) were used to determine protein (11.3, 10.7, 10.9, 11.0%), ash (0.51, 0.49, 0.52, 0.51%), and falling numbers (350, 330, 315, and 320 sec), respectively, for the four flour samples.

Rye (European cultivar Danko) was grown in the province of Limburg in Belgium. Where appropriate, rye kernels were hand-cleaned to remove foreign and broken grains.

Diethylaminoethyl cellulose (DEAE-cellulose) was obtained from Merck (Darmstadt, Germany). Catalase (1.4×10^5) was obtained from *Aspergillus niger* (Sigma, St. Louis, MO). All other chemicals were at least analytical grade.

Breadmaking

Baking absorption levels and mixing times were determined by the mixograph procedure (Finney and Shogren 1972). Breads were produced by the 10-g breadmaking procedure of Shogren and Finney (1984). Fermentation used 0.076 g of dried yeast (Fermipan, Gist Brocades, Delft, The Netherlands). The recipe contained 6.0% sucrose, 4.0% nonfat dried milk, 3.0% shortening (Crisco, Procter & Gamble, Cincinnati, OH), and 1.5% salt. The percentages were based on the flour weight at 14% moisture. Where rye extracts were tested (levels up to 4.0% of the flour weight), such extracts replaced an equal weight of wheat flour. The fermentation process (180 min, three punchings, 85% rh, 30°C) was followed by a 57-min proof under the same conditions. Breads were baked for 13 min at 213°C. All breads were produced in triplicate.

Volumes were determined by rapeseed (Shogren and Finney 1984) or glass bead (Vanhamel et al 1991) displacement. Three independent determinations with the latter method provided reasonable discrimination between items that differed in volume by at least 1 cm³. As reported previously (Vanhamel et al 1993), the total standard deviation for the 10-g breadmaking process and the volume determinations was 1 cm³.

Breads also were produced by the 100-g straight-dough breadmaking procedure of Finney (1984) under the same conditions, but with 0.76 g of dried yeast, a baking time of 24 min at 215°C, and bread volume determined by rapeseed displacement.

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Breadmaking Under Nitrogen Atmosphere

The dough mixer, along with all dry ingredients and solutions (made with previously boiled water), was placed in a large plastic bag. The air in the bag was replaced with nitrogen (three times), and the dough was mixed under a nitrogen atmosphere.

Rye Milling

The kernels were ground with a DDD President Mill (Leper, Belgium) to produce wholemeal or with a Buhler MLU-202 laboratory mill as described by Delcour et al (1989) to produce milling streams.

Extraction of the Rye Water-Soluble Extract

Procedure 1. Rye wholemeal was produced from rye kernels boiled for 90 min in ethanol (to reduce the microbial load) and then air-dried. Rye wholemeal (1.00 kg) was mixed with water (4.0 L) in a Hobart-type mixer at room temperature for 240 min. The mixture was centrifuged at $1,360 \times g$ for 30 min, then the supernatant was Buchner-filtered over paper. Two volumes of ethanol were added slowly (over a 15-min period) at room temperature to the clear extract. The precipitate was allowed to settle at 5°C overnight and was recovered by centrifugation for 10 min at $3,400 \times g$. It was dried by repeated washings with ethanol and subsequently with diethyl ether with intermediate vacuum filtration. The diethyl ether was evaporated at room temperature, and the diethyl ether odor-free extract was transferred to a closed container until used.

TABLE I
Loaf Volume of Breads Produced with North American Wheat Flour and Rye Water-Soluble (RWS) Extract Isolated According to Procedure 1

Sample No. ^a	Flour (g)	RWS (g)	Volume (%) ^b
1	10.0	0.0	100.0
1	9.9	0.1	113.0
1	9.8	0.2	118.8
1	9.6	0.4	108.7
2	10.0	0.0	100.0
2	9.8	0.2	111.0
3	10.0	0.0	100.0
3	9.8	0.2	118.8
4	10.0	0.0	100.0
4	9.8	0.2	112.0
5	10.0	0.0	100.0
5	9.8	0.2	107.0

^a Flour samples processed over six months. All values are on a 14% moisture basis.

^b Values are % of control, average of triplicates.

TABLE II
Loaf Volume of Breads Produced with North American and European Wheat Flours and Rye Water-Soluble (RWS) Extract Isolated According to Procedures 1–6

Sample No. ^a	Flour (g)	RWS (g)	Procedure	Volume (%) ^b
6	10.0	0.0	...	100.0
6	9.8	0.2	2	100.7
6	9.8	0.2	3	90.5
7	10.0	0.0	...	100.0
7	9.8	0.2	1	108.7
7	9.8	0.2	4	112.0
7	9.8	0.2	5	109.6
7	9.8	0.2	6	119.0
8	10.0	0.0	...	100.0
8	9.8	0.2	1	103.0
8	9.8	0.2	4	109.0
8	9.8	0.2	5	106.0
8	9.8	0.2	6	109.0

^a Flour samples processed over six months. Sample 6 was a North American flour. Samples 7 and 8 were European flours. All values are on a 14% moisture basis.

^b Values are % of control, average of triplicates.

Procedure 2. This procedure was the same as procedure 1, except for a 10-min boiling and subsequent cooling (to room temperature) step of the Buchner-filtered extract before the ethanol precipitation step.

Procedure 3. Material obtained in procedure 1 (5.0 g) was dissolved in 300 mL of 1.0N sodium hydroxide, flushed with nitrogen, and stored for 144 hr at room temperature. Then it was dialyzed and recovered by ethanol precipitation and the ethanol and diethyl ether washings as in procedure 1.

Procedure 4. This procedure was the same as procedure 1, except that rye wholemeal was prepared from rye kernels that were not boiled in ethanol.

Procedure 5. Instead of recovering the active components by ethanol precipitation, the clear extract was dialyzed for 48 hr in the cold room (5°C), shell frozen, and lyophilized.

Procedure 6. Procedure 5 was followed starting with rye kernels that were not boiled in ethanol.

Procedure 7. The water-soluble material was separated from rye wholemeal by shaking one portion of meal with four portions of deionized water for 4 hr at 5°C . After centrifugation (30 min , $10,000 \times g$, 4°C), the supernatant was dialyzed against deionized water (5°C , 48 hr), shell-frozen, and lyophilized.

Separation of the Water-Soluble Extract

Several techniques were used in an effort to concentrate the active factor, including anion-exchange chromatography on DEAE-cellulose, ammonium sulfate precipitation, and milling with a Buhler MLU-202 laboratory mill.

Anion-Exchange Fractionation

This technique provided sufficient quantities of the components for breadmaking trials. Before batch separation on DEAE-cellulose, the matrix was washed with HCl (0.5N), NaOH (0.5N), and preequilibrated with buffer (tris-HCl buffer, pH 8.5, ionic strength = 0.0147). The saturation point of the DEAE-cellulose was determined with dialyzed supernatant from procedure 7.

The DEAE-cellulose (resulting from 350 g of DEAE-cellulose) was added to 1,500 mL of dialyzed supernatant from procedure 7, and the mixture stirred for 60 min at 5°C . The supernatant was recovered by centrifugation ($1,520 \times g$, 15 min, 4°C). Both supernatant fractions were combined, dialyzed for 48 hr in the cold room (5°C), shell-frozen, and lyophilized. Acid proteins were desorbed with 1.0N sodium chloride at 5°C . The desorbate then was separated from the DEAE-cellulose by centrifugation at $1,520 \times g$ for 15 min (4°C), dialyzed for 48 hr in the cold room, and then lyophilized as above.

Ammonium Sulfate Precipitation

Dialyzed supernatant from procedure 7 was treated with ammonium sulfate in the cold room (5°C), and precipitated material at 30, 40, 50,

TABLE III
Loaf Volume of Breads Produced with European Wheat Flours and Rye Water-Soluble (RWS) Extract Isolated According to Procedure 7 and Further Fractionated by Anion-Exchange Chromatography into Supernatant and Desorbate

Flour ^a (g)	Isolation of RWS	RWS (g)	Volume (%) ^b
10.0	...	0.0	100.0
9.8	Procedure 7	0.2	108.7
9.975	Supernatant	0.025	100.2
9.95	Supernatant	0.05	106.2
9.9	Supernatant	0.10	111.9
9.85	Supernatant	0.15	106.2
9.8	Supernatant	0.2	102.4
9.9	Desorbate	0.1	97.5
9.8	Desorbate	0.2	91.7

^a Flour sample 9 processed over six months. All values are on a 14% moisture basis.

^b Values are % of control, average of triplicates.

60, and 70% saturations were removed immediately. After each addition of ammonium sulfate, the precipitate was allowed to form at 5°C overnight, and a centrifugation step (10,000 × g, 30 min) was performed to recover the material. It was then dissolved in deionized water, dialyzed at 5°C for 48 hr, shell-frozen, and lyophilized.

Isoelectric Focusing (IEF)

Isoelectric focusing (IEF) was performed by a Phast System with Phast Gel IEF 3-9 (43 × 50 × 0.35 mm; Pharmacia, Uppsala, Sweden). Separation conditions were as recommended by the manufacturer. Gels were silver-stained according to manufacturer (Biotechnology, Development Technique File no. 210, Pharmacia LKB).

RESULTS AND DISCUSSION

Extract Yields

Typical extract yields were 40.0 g of dry matter per kilogram of rye in procedures 1 and 2. In procedure 3, only 4.6 g of material was recovered. Where dialysis was used instead of ethanol precipitation (procedure 7), dry matter recovered was ≈45.0 g/kg of rye.

Breadmaking with Total Extracts

From breadmaking results, it was clear that the rye extract was a potent bread improver (Table I) and, at the same time, it improved the handling properties of the dough. We suspected that its effect was due to the presence of arabinoxylans. Indeed, with procedure 1, one would expect arabinoxylan material to be the most predominant component of the extract (Vinkx et al 1993). Furthermore, it is a well-known quality-determining factor in rye breadmaking (Drews 1972). However, regardless of the exact mechanism, the extract enhanced the loaf volume and improved the dough handling properties. The visual appearance of bread with added rye extract could be mistaken for bread with added oxidants in the breadmaking formula (e.g., bread corners were less sharp, and the crumb had more elongated and more uniform cells and a whiter appearance, all indicative of oxidative effects).

We next produced breads containing rye extracts isolated according to procedure 2, and we repeatedly found that the additional heating step resulted in the loss of all activity (Table II). This immediately led us to conclude that the initial ethanol boiling step (with a different solvent, intact rye kernels, and a lower temperature) had not destroyed the active component. Boiling the water-soluble extract destroyed all bread-improving effects, which strongly suggests that the improvement was not due to the presence of arabinoxylans. Indeed, it is well known that, in contrast to physiologically active proteins, arabinoxylans do not lose their physicochemical properties as a result of heating. We observed that deproteinizing the extract by an alkaline treatment (procedure 3) also resulted in all effects being lost. The fact that the bread

improver was unstable under alkaline conditions proved that it must be different from the water-insoluble rye arabinoxylans isolated earlier by Casier (1967, 1973).

These results suggest that a protein or a glycoprotein was involved in the bread improver mechanism. Accordingly, we changed our isolation procedure to make it more suited for isolation of active protein material by omitting the rye kernel boiling step (procedure 4) and/or the ethanol precipitation step (procedures 6 and 5, respectively). Based on the results presented in Table II, we then adopted procedure 7 for the remaining experiments.

Breadmaking with Anion-Exchange Fractions

The results of the breadmaking experiments are shown in Table III. Only the supernatant fraction from the DEAE-cellulose gave a volume increase, which was more pronounced at the 1% level than at the 2% substitution level. The ion-exchange treatment resulted in a volume increase by the supernatant fraction at a level 1% that was greater than that by the total fraction at a 2% level. This implies that an enrichment of the active factor had taken place in the supernatant fraction, which was enriched in alkaline protein material (pI > 7.5) as shown by IEF analysis. Therefore, we could conclude that the ion-exchange batch method is useful for enrichment of the active factor.

Breadmaking with Fraction Obtained by Ammonium Sulfate Precipitation

The fractions obtained at 30, 40, and 50% saturations of ammonium sulfate were active in breadmaking experiments (Table IV). Analysis of the fractions by IEF showed that they contained mostly basic proteins but still contained a proportion of acidic proteins. Table IV also shows clearly that these fractions at the 2% level gave a volume decrease, and that the breads were overoxidized. The breads produced with the fraction precipitated after 40% saturation, and added at the 1% level, gave the best results. A volume dosage curve was also established for the fraction precipitated with 30% saturation, which showed a maximum volume increase at the 0.5% substitution level. Therefore, these data clearly indicated that ammonium sulfate also can be an effective tool for concentration of the active protein material, and confirmed our speculation that the active material is an alkaline protein fraction.

Breadmaking with Extracts Isolated from Rye Milling Streams

Rye kernels were milled with a Buhler MLU-202 laboratory mill as described by Vanhamel et al (1991). After passage through the mill, the B- and C-fractions were recombined into one B-fraction and one C-fraction yielding 29.0 and 18.0% of milled material, respectively. Shorts and bran fractions represented 24.0 and 29.0%, respectively. The active components were isolated from these fractions according to procedure 7, and breads were pro-

TABLE IV
Loaf Volume of Breads Produced with European Wheat Flours and Rye Water-Soluble (RWS) Extract Isolated from Dialyzed Supernatant of Procedure 7 by Stepwise Addition of Ammonium Sulfate

Flour ^a (g)	(NH ₄) ₂ SO ₄ (%)	RWS (g)	Volume (%) ^b
10.0	...	0.0	100.0
9.9	30	0.1	114.7
9.8	30	0.2	80.0
9.9	40	0.1	116.6
9.8	40	0.2	92.0
9.9	50	0.1	112.7
9.9	60	0.1	95.3
9.8	60	0.2	90.0
9.9	70	0.1	96.5
9.8	70	0.2	86.9

^a Flour sample 10 processed over six months. All values are on a 14% moisture basis.

^b Values are % of control, average of triplicates.

TABLE V
Loaf Volume of Breads Produced with European Wheat Flours and Rye Water-Soluble (RWS) Extract Isolated from Rye Wholemeal or Milling Fractions According to Procedure 7

Flour ^a (g)	Milling Fraction	RWS (g)	Volume (%) ^b
10.0	...	0.0	100.0
9.9	Wholemeal	0.1	103.3
9.8	Wholemeal	0.2	106.9
9.9	B-fraction	0.1	97.6
9.8	B-fraction	0.2	101.2
9.9	C-fraction	0.1	102.7
9.8	C-fraction	0.2	103.1
9.9	Shorts	0.1	107.7
9.8	Shorts	0.2	117.6
9.9	Bran	0.1	106.2
9.8	Bran	0.2	116.0

^a Flour sample 10 processed over six months. All values are on a 14% moisture basis.

^b Values are % of control, average of triplicates.

TABLE VI
Loaf Volume of Breads Produced with North American Wheat Flours and Rye Water-Soluble (RWS) Extract Isolated According to Procedure 1 with Potassium Bromate

Flour ^a (g)	RWS (g)	KBrO ₃ (ppm)	Volume (%) ^b
10.0	0.0	0	100.0
9.8	0.2	0	107.5
10.0	0.0	10	103.4
9.8	0.2	10	112.2

^a Flour sample 12 processed over six months. All values are on a 14% moisture basis.

^b Values are % of control, average of triplicates.

TABLE VII
Loaf Volume and Crumb Grain of Breads^a with Several Additives ± Catalase

Additive	Catalase	Volume (%) ^b	Crumb ^c
None	–	100.0	U
None	+	98.2	U
Calcium peroxide, 20 ppm	–	103.0	O
Calcium peroxide, 20 ppm	+	99.2	U
Potassium bromate, 20 ppm	–	103.7	O
Potassium bromate, 20 ppm	+	104.2	O
Azodicarbonamide, 10 ppm	–	105.1	O
Azodicarbonamide, 10 ppm	+	105.4	O
Ascorbic acid, 50 ppm	–	103.8	O
Ascorbic acid, 50 ppm	+	104.2	O
Procedure 7 rye water solubles (2%)	+	112.7	O
Procedure 7 rye water solubles (2%)	–	111.4	O

^a Produced by the 100-g straight-dough breadmaking procedure of Finney (1984) from 100 g of North American wheat flour (sample 13).

^b Values are % of control, average of triplicates.

^c U = underoxidized; O = optimum.

duced with the resulting material (Table V). These results clearly showed that milling is another useful method for separating the extract into active and nonactive components. Again, the volume increases of the breads could be described in terms of oxidation effects. Shorts and bran fractions definitely contained basic proteins, as shown by IEF.

Mechanism for Improving Bread Quality

As long as the identity of the bread improver is not elucidated, it is difficult to study its mechanism. Baking results showed that its activity is additive to that of potassium bromate (Table VI) but not to that of ascorbic acid (results not shown). As can be seen from the data in Table VII, the commonly used oxidants increased the loaf volume ≈5%. This volume increase was obtained at the level of addition judged to give the optimum oxidation (crumb grain appearance). Under the same conditions, the rye extract gave a much larger loaf volume (≈12%) increase over the control. The level of addition also was judged by the optimum oxidation appearance based on the crumb grain.

We next investigated whether the rye extract improves bread dough by producing hydrogen peroxide. As shown in Table VII, the addition of catalase to the rye extract had only a minor effect on the loaf volume and crumb grain of the bread produced. We also added catalase with other commonly used oxidants and found that catalase had no effect with any of them. This indicated that none of the oxidants, including the rye extract, works through a mechanism involving hydrogen peroxide.

To determine whether the presumed enzyme in the rye extract required oxygen for its action, doughs were mixed under nitrogen. This elimination of oxygen would be expected to stop the improving effect of the rye extract, if the presumed enzyme was an oxidase. Mixing the dough under nitrogen gave a greatly decreased loaf volume and a very underoxidized appearance (Table VIII). This suggests that the presumed protein may be an oxidase. In addition, the reduction of volume to a value much lower than that

TABLE VIII
Loaf Volume of Breads Produced with North American Wheat Flours and Rye Water-Soluble (RWS) Extract Isolated According to Procedure 7 Mixed Under Air or Nitrogen Atmosphere

Flour ^a (g)	RWS (g)	Atmosphere	Volume (%) ^b
10.0	0.0	Air	100.0
9.8	0.2	Air	109.1
10.0	0.0	Nitrogen	89.6
9.8	0.2	Nitrogen	89.6

^a Flour sample 13 processed over six months. All values are on a 14% moisture basis.

^b Values are % of control, average of triplicates.

of the control containing no rye extract suggested that oxygen itself has a large effect, as suggested previously by Bloksma and Bushuk (1988).

CONCLUSIONS

Rye water-solubles contain an agent, presumably of proteinaeous nature, that improves not only the handling properties of wheat flour dough, but also the volume of the resulting bread. The evidence that the bread improver is a protein or glycoprotein rather than a pentosan includes the fact that its activity is destroyed upon boiling and does not withstand an alkaline treatment. Furthermore, the most active fractions were enriched in alkaline protein material. Although its effect is additive to that of potassium bromate, it is not additive to that of ascorbic acid, another feature that rules out arabinoxylans as active factors. Of further importance is that this rye protein produces effects comparable to those of commonly used bread oxidants.

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