Protein Alteration in Flour Damaged by Ball-Milling and Roller-Milling[1]

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ABSTRACT

Protein alteration in flour milled with varying degrees of severity by ball and roller mills was studied by means of nonprotein nitrogen (NPN) determinations, specific color reactions, Sephadex column chromatography, gel electrophoresis, and sulfhydryl group analysis. The proteins of the water-soluble fraction were studied most extensively. The possibility of protein denaturation in highly damaged flour was indicated by a decrease in the total flour nitrogen contained in the water-soluble fraction. An increase in NPN in the water-solubles was observed as severity of milling increased. Two color reactions for measurement of amino groups supported the NPN results. In addition, the water-soluble proteins produced two main peaks when separated on a Sephadex G-50 column. Average values for several column determinations indicated a slight increase in the lower-molecular-weight protein content of the damaged flour. These data indicate an alteration of the protein structure. Gel electrophoresis of water-soluble proteins revealed 12 components with no extreme differences between undamaged and damaged samples. Likewise, the –SH content did not show appreciable quantitative differences.

The overgrinding of flour, its effects on flour quality, and, in particular, the effect on the starch fraction have been studied by various workers (1–8). In the past, the properties of flour affected by overgrinding have been attributed primarily to the starch fraction. Only a few authors mention the effects of overgrinding on the protein components of flour.

Alsberg and Griffing (1) were among the first to discuss the effects of fine grinding on the properties of flour. They concluded that gluten quality was injured with overgrinding, as evidenced by the feel and appearance of the gluten. No single piece of evidence was given, however, which proved that grinding in itself altered gluten directly. Atkinson and Fuehrer (6) also discussed the effects of overgrinding on flour characteristics; whether the changes effected by overgrinding were due to the action upon the starch or to alteration in the protein structure was not established. They believed that both aspects were undoubtedly involved to various degrees. Schlesinger (8) concluded from his study that the mechanical action was not sufficient to alter the protein structure. This work will describe certain changes in the biochemical properties of the proteins which resulted from overgrinding.

MATERIALS AND METHODS

Flour

A commercial bakers' hard red spring patent flour (14.0% protein, d.b.) was used throughout this study. The flour was unbleached and untreated. To

[1]Presented at the 51st annual meeting, New York, N. Y., April 1966. Published with the approval of the Director of the Agricultural Experiment Station, North Dakota State University, Fargo, North Dakota, as Journal Series No. 98. Taken in part from a thesis submitted by B. L. D'Appolonia to the North Dakota State University, in partial fulfillment of requirements for the M.S. degree.

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facilitate calculation of material balances, the data in this study were reported on dry basis (d.b.).

Damaged Samples

Ball-milling was performed in a stoneware jar with small polished stones as the grinding agent. The mill was electrically driven at 25 r.p.m. by means of a small motor. Samples of flour (300 g.) were ball-milled at room temperature for periods of 4, 8, 16, and 24 hr. and for 24 hr. at 5°C. Roller damage was created by passing the flour through the reduction rolls of an Allis-Chalmers mill. The spacing between the rolls was gradually reduced from 0.003 in. for the first pass to a touching position of the two rolls during the last pass. Samples representing four, six, eight, and ten passes through the rolls were collected.

Flour Fractionation

The flour was fractionated into four components (starch, sludge, gluten, and water-solubles) according to the method of Gilles, Kaelble, and Youngs (9). The flour was not defatted before fractionation, nor was the fractionation carried out under an atmosphere of nitrogen. In the majority of flours, only the water-soluble fraction was retained for further analysis.

Measurement of Nonprotein Nitrogen

Tungstic acid was used as protein precipitant to determine the nonprotein nitrogen (NPN) in the water-solubles extracted from the undamaged and damaged flour. The method was a modification of the work by Bell (10). Freeze-dried water-solubles (0.5 g.), of known nitrogen content, were dissolved in distilled water (20 ml.). Twenty milliliters of a mixture containing 8 parts 0.08 *N* sulfuric acid and 1 part 10% aqueous sodium tungstate solution was added. The precipitate was centrifuged at 10,000 $\times$ *g* and the percent nitrogen in the supernatant was determined after 20- and 60-min. centrifugation periods. All nitrogen values were determined by the Kjeldahl procedure (11).

Starch Damage Determination

Starch damage in the various flours was measured according to the method of Medcalf and Gilles (12).

Measurement of Amino Groups

2,4,6-Trinitrobenzene 1-sulfonic acid was used to measure amino groups in the water-solubles. The method was that of Satake *et al.* (13), adapted to freeze-dried water-solubles as follows: 2 ml. of sample solution, which contained the freeze-dried water-solubles, was mixed with 2 ml. of 4% sodium bicarbonate and 2 ml. of 0.10% 2,4,6-trinitrobenzene 1-sulfonic acid and placed in an oven at 40°C. for 2 hr. In all determinations the 2 ml. of sample solution contained 0.15 mg. of nitrogen. After 2 hr., 2 ml. of 1*N* HCl was added. The solution was centrifuged until clear and the absorbance measured at 350 mμ in a Spectronic 20 colorimeter.

Figure 1 shows a standard curve prepared for this reaction with glycine as the standard. The results were then expressed in terms of μmoles glycine per 0.15 mg. nitrogen.

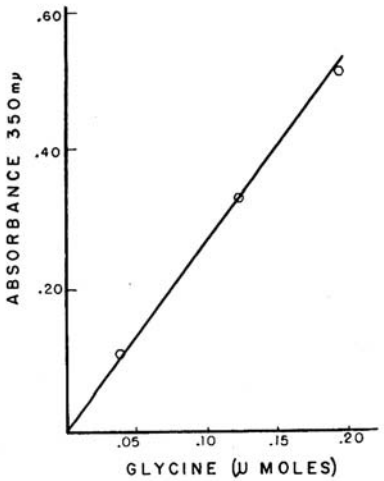


Fig. 1. Standard curve for glycine using 2,4,6-trinitrobenzene 1-sulfonic acid reagent.

Sephadex Gel Filtration

A 1×50-cm. column was used, with Sephadex G-50 Fine to pack the column. The swelling of the gel, packing of the column, and application of the sample were performed essentially as described by Flodin (14). One milliliter of a solution of the freeze-dried water-solubles was layered on the gel and allowed to enter the bed. The solution of water-solubles applied to the column contained 8.88 mg. of protein for the undamaged flour and 9.35 mg. of protein for the damaged flour. A flow rate of 1 ml./min. was maintained. The column eluant was a solvent system consisting of urea, acetic acid, and hexadecyltrimethylammonium bromide prepared according to the procedure of Meredith and Wren (15). Two-milliliter fractions were collected with the use of an automatic fraction collecter. The absorbance of each fraction was measured at 280 and 260 mμ. According to the formula devised by Kalckar (16), an estimation of protein content in the presence of nucleic acid was obtained.

Gel Electrophoresis

The polyacrylamide gels were prepared as described by Raymond and Wang (17). The gel was cut in the center and the sample inserted by means of 5-mm. × 12-mm. filter paper strips soaked in a 5% protein solution of the water-solubles. The buffer used in the horizontal electrophoretic apparatus was 0.05*M* tris citrate, pH 8.0. The voltage gradient, measured across 20 cm. of the gel, was 5.5 volts/cm. Duration of the analysis was 3.5 hr.

Bands were detected by staining with 0.1% Amido Schwartz in methanol:water:acetic acid (5:5:1) for 30 min. and destaining in this solvent. The gels were then soaked for 24 hr. in 20% glycerol to prevent shrinkage and drying out during densitometer measurements. This also returned the gels to their original size. A Photovolt densitometer, with the recorder set on logarithmic response, was used to measure the intensity of the bands.

Sulfhydryl Determination

The apparatus used for the sulfhydryl determination was similar to that described by Kolthoff and Harris (18). The titration was performed according to Sokol *et al.* (19). For the highly damaged flours, a thick paste was made first in order to achieve proper dispersion and avoid clumping.

RESULTS AND DISCUSSION

The mechanical action exerted by a ball mill on flour is not identical to that of a roller mill. Ball-milling exerts a pounding action which readily damages flour. A shearing, crushing action takes place with a roller mill.

TABLE I
YIELDS AND NITROGEN VALUES OF VARIOUS FRACTIONS OF A COMMERCIAL BAKERS' PATENT FLOUR

FLOUR TYPE	FRACTION	YIELD	NITROGEN IN FRACTION[a]	NITROGEN IN FRACTION AS FUNCTION OF TOTAL N IN FLOUR
		%	%	%
Undamaged[b]	Gluten	13.8	11.8	66.5
	Starch	49.2	0.10	2.0
	Sludge	23.8	1.0	9.7
	Solubles	5.7	4.6	10.7
Ball-milled[c] 24 hr. at room temp.	Gluten	13.0	13.2	70.0
	Starch	46.1	0.10	1.9
	Sludge	26.5	0.54	5.8
	Solubles	6.9	3.5	9.9
Ball-milled[b] 24 hr. at 5°C.	Gluten	12.2	13.3	66.2
	Starch	47.1	0.10	1.9
	Sludge	27.3	1.0	11.1
	Solubles	7.7	3.5	11.0

[a] Expressed on dry basis.
[b] Values reported are an average of two fractionations.
[c] Single determination.

Table I gives the percent yield and nitrogen values of the four fractions obtained on fractionation of undamaged flour and flour ball-milled for 24 hr.

A decrease in the percent recovery of the starch fraction with the ball-milled flour indicates that some starch was highly damaged. The damaged starch was collected in the water-soluble fraction if solubilized and in the sludge fraction if not solubilized.

The proteins of the water-soluble fraction were the main interest in this work. Table II shows the yield and nitrogen results on the water-soluble fraction extracted from the undamaged flour and a series of damaged flours. As damage to the flour increases, the percent of water-solubles recovered also increases. For the ball-milled flours, the total flour nitrogen contained in the water-solubles decreases as damage to the flour increases. This decrease may be attributed to the protein's being denatured and becoming insoluble. The heat developed during ball-milling is a possible cause of this denaturation. This is suggested by the fact that the total flour nitrogen contained in the

TABLE II
YIELDS AND NITROGEN VALUES OF WATER-SOLUBLES

FLOUR TYPE	YIELD[a]	N[a]	N/TOTAL N
	%	%	%
Undamaged	6.07	4.60	11.39
Ball-milled at room temp.: 4 hr.	6.62[b]	4.16[b]	11.24
8 hr.	6.66	3.98	10.82
16 hr.	7.04	3.68	10.57
24 hr.	7.23	3.37	9.95
Ball-milled at 5°C. 24 hr.	7.31	3.52	10.50
Roller damage: four passes	6.92[b]	4.40[b]	12.43
six passes	7.33	4.09	12.24
eight passes	7.29	3.92	11.66
ten passes	7.37[b]	3.74[b]	11.25

[a] Values reported are an average of two or more fractionations.
[b] Single determinations.

water-solubles is somewhat higher in the flour ball-milled for 24 hr. at 5°C. compared to the flour ball-milled 24 hr. at room temperature.

The total flour nitrogen contained in the water-solubles of the roller-damaged samples increased with minor amounts of damage. However, this value decreased as damage became more severe. It is possible that a minor amount of damage caused some protein to become soluble.

Nonprotein Nitrogen

Table III gives the NPN results obtained with tungstic acid as protein precipitant and different centrifugation times. Starch damage values for the different flours also are listed. The results for NPN are expressed as a percent of total water-soluble nitrogen and also as a percent of total flour nitrogen. The NPN in the water-solubles increased as damage to the flour increased. The starch damage values reflect the extent of damage to the various flours.

TABLE III
COMPARISON OF STARCH DAMAGE AND NPN VALUES

FLOUR TYPE	STARCH DAMAGE	NPN IN WATER-SOLUBLE NITROGEN: Centrifugation Period 20 Min.[a]	60 Min.[b]	NPN IN TOTAL FLOUR NITROGEN: Centrifugation Period 20 Min.	60 Min.
	%	%	%	%	%
Undamaged	8.6	14.5	10.7	1.6	1.2
Ball-milled: 8 hr.	23.0	16.8	12.4	1.7	1.3
16 hr.	28.7	17.5	13.3	1.8	1.4
24 hr.	37.7	23.0	14.3	2.2	1.4
24 hr. (5°C.)	37.7	21.7	14.1	2.2	1.5
Roller damage: four passes	10.7	16.4	10.2	2.0	1.3
six passes	14.3	17.0	11.7	2.1	1.4
eight passes	15.1	19.8	12.3	2.4	1.5
ten passes	21.0	20.9	11.9	2.4	1.3

[a] Average of four or more determinations with tungstic acid as protein precipitant.
[b] Average of two or more determinations with tungstic acid as protein precipitant.

As the centrifugation period was increased, the difference in NPN between undamaged and damaged samples was not so great. However, a progressive increase as damage to the flour increased was still evident. The explanation may be twofold. In one case, the protein may be altered by an unfolding of the backbone chain due to rupture of weak bonds involved in the secondary and tertiary structure, which causes the protein to sediment at a slower rate. Concomitantly, there appears to be a rupture of disulfide or peptide bonds. This results in an increase in lower-molecular-weight material, which explains the increase in NPN.

A further experiment was made in which soluble starch extracted from ball-milled starch was added to the water-solubles extracted from the undamaged flour. The amount added corresponded to the increased yield of water-solubles extracted from the 24-hr. ball-milled flour. The same NPN values were obtained for this sample as in previous determinations. This indicated that the material which became soluble with the damaged flours was not affecting the NPN values. Two other protein-precipitating agents, phosphotungstic acid and trichloroacetic acid, gave NPN results which supported the values shown in Table III. Even though the NPN values themselves were different, the same relative differences were observed.

Measurement of Amino Groups

Table IV gives the results obtained when the reagent 2,4,6-trinitrobenzene 1-sulfonic acid was used to measure primary amine groups. As damage to the flour increased, an increase in amino groups was observed. A possible

TABLE IV
AMINO GROUP ANALYSIS OF WATER-SOLUBLES

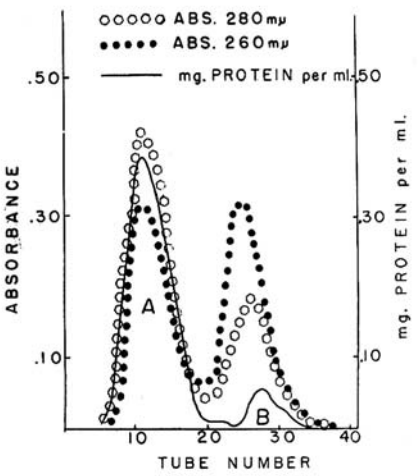
Flour Type	Absorbance at 350 mμ[a]	Glycine	Flour Type	Absorbance at 350 mμ[a]	Glycine
		μmoles/0.15 mg. water-sol. N			*μmoles/0.15 mg. water-sol. N*
Undamaged	0.45	0.165	Roller damage		
Ball-milled: 4 hr.	0.47	0.175	four passes	0.49	0.180
8 hr.	0.50	0.185	six passes	0.47	0.175
16 hr.	0.52	0.192	eight passes	0.49	0.180
24 hr.	0.54	0.200	ten passes	0.50	0.185
24 hr. (5°C.)	0.54	0.200			

[a] Average of two or more determinations.

explanation for this observation is that certain reacting groups which were buried inside the helical structure of the protein became exposed for reaction. Another possibility, as previously mentioned for the increase in NPN values, is the splitting of peptide bonds. This increase in amino groups as damage to the flour increased also was shown when the ninhydrin color reaction was used.

Sephadex Gel Filtration

Figures 2 and 3 show typical gel-filtration curves obtained for the water-solubles extracted from undamaged and 24-hr. ball-milled flour.



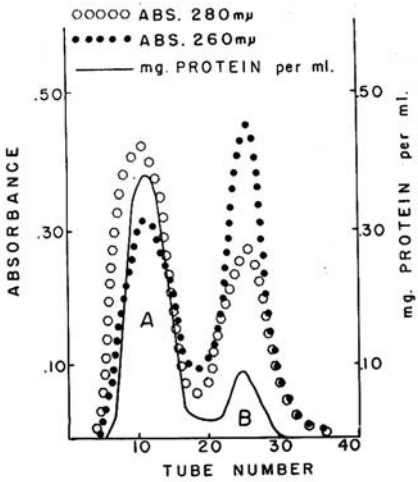


Fig. 2 (left). Column chromatogram of water-solubles extracted from an undamaged flour. The 1×50-cm. column was packed with Sephadex G-50 Fine, with a solvent system consisting of urea (3*M*), acetic acid (0.1*M*), and hexadecyltrimethylammonium bromide (0.01*M*), dissolved in water as column eluant.

Fig. 3 (right). Column chromatogram of water-solubles extracted from a 24-hr. ball-milled flour. The 1×50-cm. column was packed with Sephadex G-50 Fine, with a solvent system consisting of urea (3*M*), acetic acid (0.1*M*), and hexadecyltrimethylammonium bromide (0.01*M*), dissolved in water as column eluant.

TABLE V
GEL FILTRATION OF WATER-SOLUBLES

FLOUR TYPE	PEAK A[a]	PEAK B[a]	FLOUR TYPE	PEAK A[a]	PEAK B[a]
	%	%		%	%
Undamaged	89.6	10.4	Ball-milled 24 hr.	84.8	15.6
	84.7	15.3		87.5	12.5
	88.1	11.9		87.6	12.4
	88.0	12.0		83.7	16.3
Average	87.6	12.4	Average	85.4	14.2

[a]The ratios of the designated peak area to the total area expressed as percent.

Table V gives the area under the two main peaks, A and B, as a function of the total area for four column determinations on each of the water-soluble fractions. The area measured was that represented by the formula:

$$1.45\ \text{absorbance}_{280} - 0.74\ \text{absorbance}_{260}$$

The average of the four determinations shows a slight increase in the lower-molecular-weight area for the water-solubles extracted from the damaged flour. These results support the data from the NPN and amino group experiments.

Gel Electrophoresis

Figure 4 illustrates a typical electrophoretic pattern obtained from the water-solubles extracted from an undamaged flour with the bands detected by

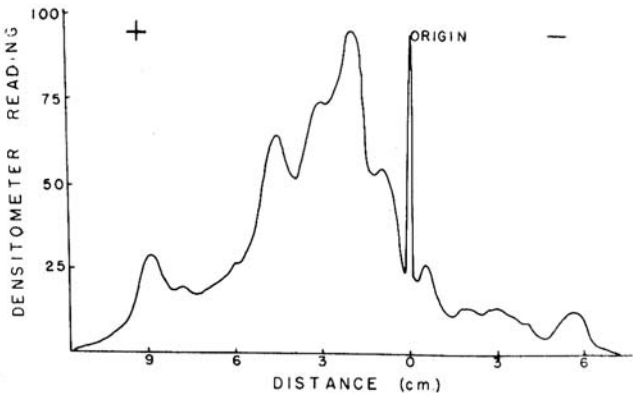


Fig. 4. A densitometric pattern of water-solubles extracted from an undamaged flour, showing relative amounts of the different components after subjection to gel electrophoresis.

densitometry. Although the pattern obtained with water-solubles extracted from the undamaged and damaged flour revealed 12 components, slight changes in the shape of certain peaks were observable.

Sulfhydryl Groups

The different samples all gave a sulfhydryl content of 0.88 μeq./g. of flour. The results indicate that no apparent increase in the sulfhydryl content of flour occurred with damage by overgrinding under these conditions.

SUMMARY

An alteration in protein structure with overgrinding of a flour has been suggested by the results of a number of experiments.

These results include: the decrease in the total flour nitrogen contained in the water-solubles; an increase in NPN; an increase in amino groups; an increase in lower-molecular-weight protein as shown by gel-filtration experiments; and a slight variation in the shape of the peaks obtained in gel electrophoresis.

Acknowledgment

The authors acknowledge the courtesy of the North Dakota State Mill and Elevator, Grand Forks, in supplying the flour sample.

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[Received March 31, 1966. Accepted December 13, 1966]