Glutathione in Wheat Flour. II. An Enzymatic Determination

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ABSTRACT

The glutathione reductase method was used for determining glutathione in wheat flour. The content of glutathione was 2.4 and 1.4 μeq./100 g. flour from hard red winter and hard red spring wheat respectively. An acidic fraction of the dialysate separated by gel filtration on DEAE-Sephadex with 1*M* acetic acid was found to contain glutathione and some other peptides. Half of the glutathione in the original dialysate was detected in the acidic fraction of DEAE-Sephadex by the enzymatic method.

The presence of glutathione in wheat flour was postulated in 1940 by Sullivan, Howe, Schmalz, and Astleford (1). The progress of cereal chemistry indicates the greater significance in its possible presence. The dough-improving effect of ascorbic acid (2,3) and the sulfhydryl-disulfide interchange reaction (4) may involve glutathione in flour. The present authors used the alloxan method for estimating glutathione (5). However, the amount which they obtained could not be considered as the real content of glutathione, since the specificity of this method for glutathione has not been established completely. Proskuryakov and Zueva (6) showed the presence of low-molecular SH and S–S compounds, 0.7 to 0.99 μeq./g. of flour, by the EDTA-urea method.

The present authors employed the enzymatic method utilizing glutathione reductase (7) for determining glutathione, because of the specificity and sensitivity of this method.

MATERIALS AND METHODS

A hard red winter wheat, Warrior, and a hard red spring wheat, Marquis, which had been grown in 1965 and stored in a nitrogen atmosphere, were milled in a test mill. The first-middlings were used as samples. Yields were 37.0 and 41.5% respectively. They contained 12.3 and 13.3% of protein, 0.33 and 0.42% of ash, and 13.3 and 12.8% moisture respectively.

Reagents

Sodium nicotinamideadeninedinucleotide phosphate ($NADPH_2$) was purchased from Mann Research Laboratories, Inc., New York, N.Y. 5,5′-Dithiobis-(2-nitrobenzoic acid)(DTNB) was obtained from the Aldrich Chemical Co. Inc., Milwaukee Wis.; glutathione, in reduced form, from Kirin Brewery Co., Tokyo, Japan. Glutathione, in oxidized form, was purchased from Sigma Chemical Co., St. Louis, Mo. Glutathione reductase was obtained from C. F. Boehringer & Soehne, Mannheim, Germany. Sodium ethylenediaminetetraacetate (EDTA) was the product of Wako Pure Chemical Co., Osaka, Japan.

Fractionation and Identification

The flour extract was obtained by centrifuging a flour suspension made by mixing 300 g. flour and 750 ml. water in a Waring Blendor. The extract (400 ml.) was dialyzed against 1,400 ml. of deionized water. The dialysate

was evaporated to 3 ml. at 25°C. *in vacuo* (N content 10.5 mg./ml.) and used for the fractionation and thin-layer chromatography experiments.

The DEAE-Sephadex A-25 medium, from Pharmacia, Uppsala, Sweden, was allowed to swell in a large volume of water and decanted to remove small particles. It was purified by successive treatments with 0.5*N* hydrochloric acid and 0.5*N* sodium hydroxide. The purified resin was buffered in 0.1*M* acetic acid and was washed. All the pretreatment was done in a beaker by the batch method. It was packed in a column, 30 mm. in diameter and 350 mm. long. An aliquot of the concentrated dialysate (3 ml.) was put on the column. Stepwise fractionation was done with 550 ml. distilled water, 1,750 ml. of 0.1*M* acetic acid and 650 ml. of *M* acetic acid. Each 5 ml. of fraction was collected and tested for ninhydrin reaction (8) and for pH (Hitachi-Horiba Model M-4 pH meter). The same procedure was followed with standard glutathione solution (28 mg./4 ml.) as a control experiment.

The fraction which might contain acidic peptides in the eluate (2,550–2,675 ml.) (Fig. 1) was collected and dried at 25°C. *in vacuo*. The dried substance, called peak III, was dissolved in 7 ml. of deionized water.

A 0.3-mm. film of Silica Gel G (Merck Co., Germany) was made on a glass plate (20 × 20 cm.^{2}) by flowing the gel suspension (gel:water, 1:2) and allowing it to dry in an oven at 100° C. One-half of the concentrated peak III was oxidized with performic acid, hydrolyzed with 6*N* hydrochloric acid (9), and evaporated *in vacuo* over calcium oxide in a desiccator. The untreated and concentrated peak III, the oxidized and hydrolyzed peak III, and standard samples—glutamic acid, glycine, cysteic acid, and glutathione—were applied on the silica gel plate. They were developed in a jar containing a mixture of n-butanol, acetic acid, and water (2:1:1, v./v.) for 60-90 min. until development of the liquid was 10 cm. A 1% ninhydrin solution in butanol was sprayed to locate the compounds.

Assay of Glutathione by Enzymatic Method with Glutathione Reductase

The principle of this method is based on the fact that the reduction rate of DTNB in the presence of glutathione reductase and $NADPH_2$ is proportional to the small quantities of glutathione present. Initially the reductase was diluted with distilled water, so that a change in extinction occurred at 412 mμ of around 0.02/min. with 0.5 γ of glutathione in the medium described in the following paragraph.

Preparation of Standard Curve. A 0.2 ml. of glutathione solution containing 0.1 to 2.0γ was added to a cuvet containing 2.5 ml. of 0.05*M* phosphate buffer (pH 7.1), 0.8 ml. of 1*M* EDTA and 0.03 ml. of DTNB solution (39.6 mg. of DTNB and 15 mg. of $NaHCO_3$ per 5 ml. of 0.1*M* phosphate buffer, pH 7.0). The cuvet was shaken, 0.1 ml. of suitably diluted enzyme was added, and 2 min. later 0.1 ml. of $NADPH_2$ solution (4 mg./ml. of 0.02*N* NaOH). The cuvet was shaken again, the time recorded, and the uniform change in extinction at 412 mμ/min. noted from the 1st to the 6th min. The experiment was repeated with various amounts of glutathione. The E_{412} time line was written at each glutathione concentration. A standard curve was obtained by plotting the slope of the E_{412} time line against the concentrations of glutathione.

Determination of Glutathione in Flour

The dialysate of aqueous extract of flour was prepared by dialyzing a flour suspension (50 g. flour in 150 ml. water) against 150 ml. of distilled water in a Visking tube under constant stirring for 24 hr. at 5°C. The dialysate was concentrated *in vacuo* to 18 ml./50 g. flour. It was analyzed for glutathione by the enzymatic method as indicated previously. The concentrated peak III, which was prepared by fractionation on DEAE-Sephadex column by the same procedure as shown in the previous section, was also tested. The glutathione content was obtained by measuring the slope of the E_{412} time line on the standard curve.

RESULTS AND DISCUSSION

Isolation of Acidic Component of DEAE Sephadex Column

The results of fractionation from the DEAE Sephadex A-25 in which the dialysate of flour extract was applied, is shown in Fig. 1. The sharp peak III was detected in the third elution with 1*M* acetic acid. In a separate experiment with standard glutathione, a peak of ninhydrin-positive material appeared at the position similar to that of peak III, as indicated by the arrow

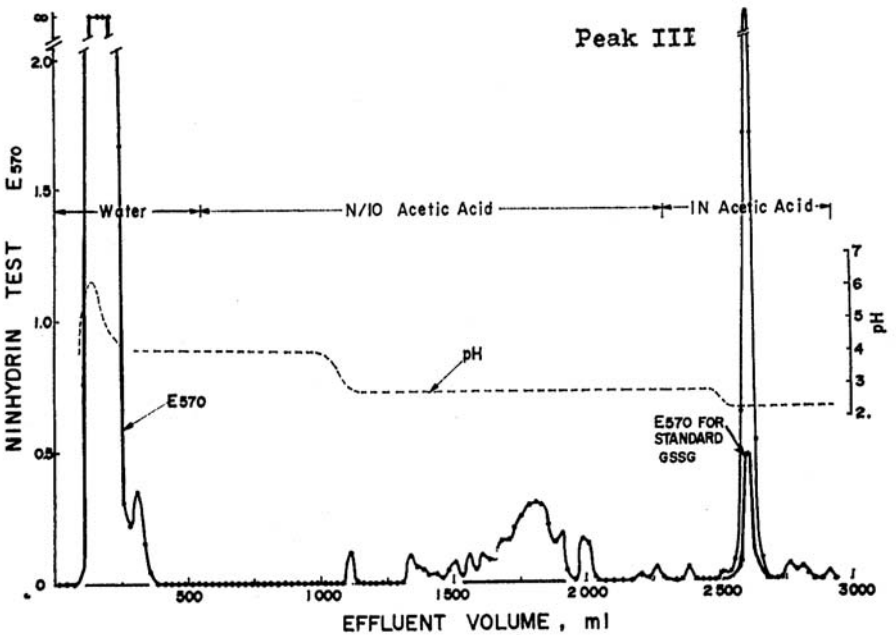

Fig. 1. Fractionation of the dialysate of the flour extract on DEAE-Sephadex A-25 column.

in the figure. If the glutathione is present in the flour extract, it would be found in this fraction. The other peaks in the elution with water and 0.1*M*

acetic acid are not discussed here since glutathione is the only substance identified in this study.

Identification of Acidic Substances in Peak III

The chromatograms of the untreated fraction peak III and the oxidized and hydrolyzed peak III are shown in Fig. 2 with those of standard samples.

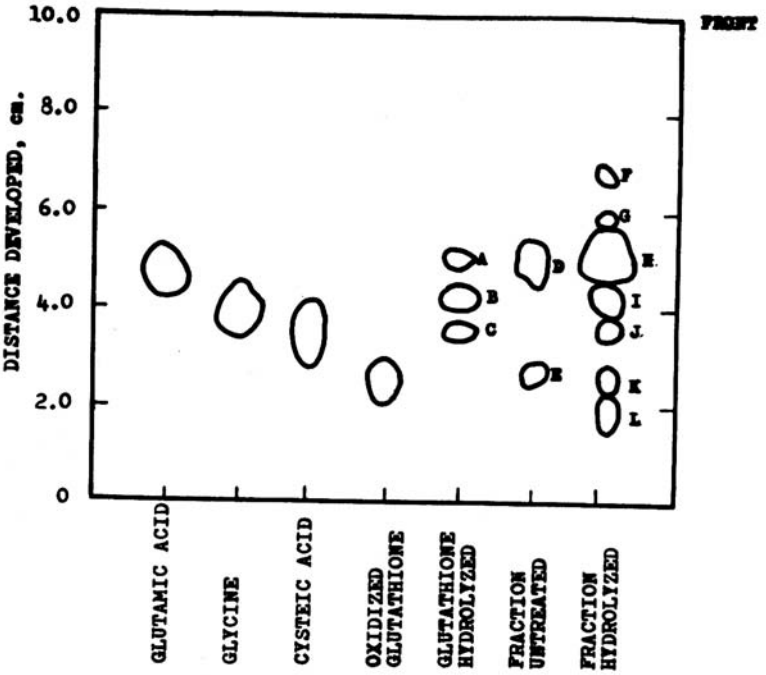

Fig. 2. Thin-layer chromatogram of the acidic fraction (peak III) of the dialysate, the oxidized and hydrolyzed fractions, and standard substances.

The eluate was found to contain two components, D and E; spot D, whose R_f is 0.5, seems, on comparison with the R_f's of the standards, to be glutamic acid. Spot B is probably glutathione. Seven spots were located after hydrolysis. H, I, and J have nearly the same R_f values as glutamic acid, glycine, and cysteic acid respectively. They may correspond to three spots, A, B, and C, from the standard glutathione hydrolysate.

It is difficult to determine whether spot K is unreacted glutathione or another compound. Small spots F, G, and L would indicate the presence of some acidic peptides other than glutathione which is located at the same position as those of the two original components. This is supported by the fact that the strength of color intensity of the untreated fraction (peak III) with ninhydrin was ten times that of the probable amount of glutathione in flour.

Estimation of Glutathione in Dialysate of Flour Suspension and in Fraction with 1M Acetic Acid on DEAE-Sephadex

The enzymatic method for determining glutathione was used with the samples described previously. The results, recalculated from the values obtained for the dialysate, are summarized in the table below.

Flour	*Total Glutathione μeq./100 g. flour*
Warrior (HRW)	2.4
Marquis (HRS)	1.4

The amount shown in the table is reliable on the basis of specificity of the enzymatic method. However, more than this amount must be present in the flour if one considers the glutathione bound with protein through the S–S exchange reaction (4).

The flours tested contained 0.3–0.4% ash and thus were contaminated very little with germ or bran. The data should be applicable to commercial flours. The difference in glutathione content found between winter and spring wheat flours may be due to the difference in the class or variety of wheat or to other variations.

The amount of glutathione obtained in this experiment is extremely small as compared with the results found by the alloxan method (5) and by Proskuryakov and Zueva (6). Some sulfhydryl compounds other than glutathione might be estimated as glutathione by those authors.

Fifty percent of glutathione in the original dialysate was recovered in the acidic fraction of the dialysate on DEAE-Sephadex.

The amount of glutathione determined by the enzymatic method represents the total, including oxidized and reduced glutathione. The total amount is significant in dough, since redox enzymes may be present in flour (2,3,6,10). Our previous paper (5), however, indicated that glutathione is present as the oxidized form in aged flour.

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