HISTOCHEMICAL CHARACTERIZATION OF WHEAT AND WHEAT PRODUCTS

V. Sulfhydryl Groups: Their Localization in the Wheat Kernel[1]

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ABSTRACT

The method of Barrnett and Seligman for histochemical visualization of protein-bound –SH groups in animal tissue cells has been applied to localize sulfhydryl groups in the wheat kernel. Wheat sections, fixed in 2% trichloroacetic acid in ethanol, were reacted with a buffered solution of 2,2′-dihydroxy-6,6′-dinaphthyl disulfide. The colorless, insoluble oxidation product was monoazotized to form a colored violet dye at sites of protein-bound sulfhydryls. Major sites of –SH groups were the aleurone layer and the germ. The histochemical method was specific and inhibited by oxidizing or –SH-blocking reagents.

Despite the importance of the sulfhydryl and the disulfide groups in flour proteins, their determination has been difficult. Nearly all methods for the estimation of sulfhydryl groups of proteins (1,3) are nonspecific, because functional groups other than sulfhydryl react with the reagents.

Barrnett and Seligman (2) have used a number of histochemical methods to demonstrate sulfhydryl groups. To improve the sensitivity and specificity of sulfhydryl histochemistry, they developed a new reagent, 2,2′-dihydroxy-6,6′-dinaphthyl disulfide (DDD). The reagent contains a disulfide linkage, a specific oxidizing group, and a naphthol moiety for coupling, to form an azo dye. The basis for using this compound depends on specific oxidation of sulfhydryls at an alkaline pH. The reaction involves, in addition to reduction oxidation, transfer of the chromogenic naphthol moiety to protein and formation of an oxidation product, from which a colored compound is produced. Barrnett and Seligman suggested producing an azo dye by coupling with tetra-azotized diorthoanisidine. Other monocoupling diazonium salts have been proposed by Cafruny *et al.* (4) and by Teiger *et al.* (7).

The reaction between DDD and protein-bound –SH groups is specific (1). The staining reaction in animal tissues was inhibited by oxidizing the –SH groups with dilute iodine and by using blocking agents such as N-ethylmaleimide and iodoacetate.

Hyde and Paliwal (6) recently tested the applicability of the Barrnett-Seligman (2) method to plant material. N-ethylmaleimide

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was found to be a reliable thiol-blocking reagent. The DDD reaction also could be partly blocked by 0.1*M* *p*-chloromercuribenzoate, but the blockage was partly overcome if the DDD reaction was continued a long time. The reaction was blocked with neither iodine nor iodoacetate.

Sulfhydryl groups are known to be important in animal tissues (1,5) and in plant tissues (6).

The purpose of this investigation was to demonstrate histochemically the presence and distribution of sulfhydryl groups in the wheat kernel and, if possible, to use the information obtained from histochemical studies to determine sulfhydryl groups in wheat flour. Localizing the sulfhydryl groups histochemically in the wheat kernel also provides the possibility of revealing particles originating from sulfhydryl-rich tissues, in wheat flour.

Materials and Methods

Materials. Selected, sound kernels of hard red winter wheat or of dent corn were cut at the distal end and kept overnight in 2% trichloroacetic acid (TCA) in 80% ethanol solution. The kernels were cut into transverse sections about 20μ thick for microscopic observation with transmitted light, and into sections 50μ thick for observation were reflected light. Materials included were:

Wheat germ, wheat bran, and wheat flour fixed overnight in 2% TCA in 80% ethanol
2,2′-dihydroxy-6,6′-dinaphthyl disulfide (DDD)[2]
Tetra-azotized diorthoanisidine[3]
Fast Blue RR[4]
0.1% aqueous solutions of potassium bromate, potassium iodate, potassium iodosobenzoate, and N-ethylmaleimide[5]
Saturated water solution of potassium periodate
0.1*M* Michaelis barbital buffer, pH 8.5
Dilute acetic acid, pH 4.0–4.5
0.067*M* Sörensen phosphate buffer, pH 7.4
Ethanol: 95, 70, 50, and 10% (water) solutions
Diethyl ether

Methods. When used in excess at pH 8.5, DDD (I, Fig. 1) forms, with active sulfhydryl groups of fixed tissue proteins, a colorless substance (II) which can be converted to an intensely colored azo dye (IV) by a coupling reaction. The colorless oxidation product (II) is insoluble in water and diethyl ether, so that an excess of DDD and the reaction by-product (III) can be washed from the tissues with organic solvents. If coupling is carried out with tetra-azotized diorthoanisidine, either a red color (due to monocoupling) or a blue color (due to di-

[2] From Schwartz Laboratories, Mount Vernon, N. Y.
[3] From Schwartz Laboratories, Mount Vernon, N. Y.
[4,5] From Dajac Laboratories, Philadelphia, Pa.

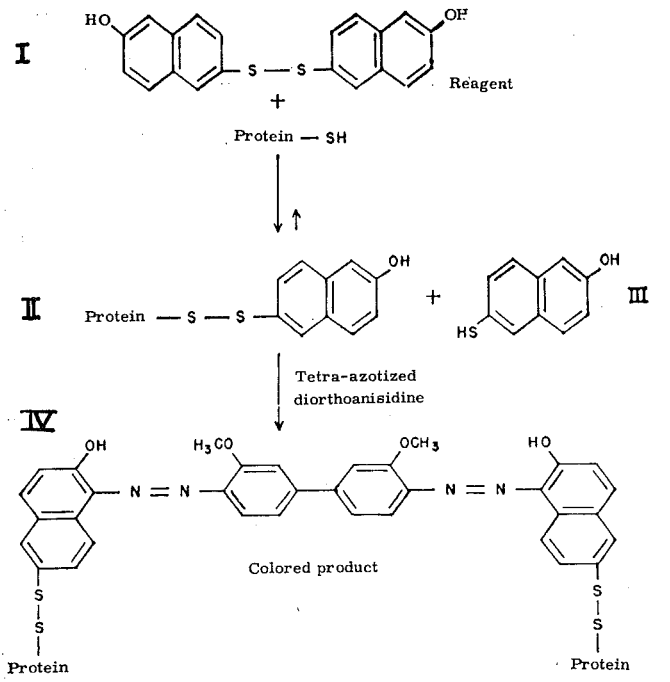

Fig. 1. Scheme of the Barrnett-Seligman histochemical procedure for determination of thiol groups. (Science **116**: 323–327; 1952. Reprinted by permission.)

coupling) develops at the sites of protein sulfhydryl groups (IV). The mechanism of the staining procedure is outlined in Fig. I.

Preliminary experiments were made with tetra-azotized diorthoanisidine. Though results were satisfactory, the reagent was replaced with the Fast Blue RR monocoupler recommended by Cafruny *et al.* (4). This was done to obtain a single-colored (violet-red) product of monocoupling, as it was desired to use the information obtained from histochemical tests for the quantitative determination of –SH groups in flour.

Wheat sections were mounted on slides with minimal albumin for the staining procedure. The albumin did not interfere with the histochemical reaction. The slides were incubated 1 hour at 50°C. in a filtered alcohol buffer mixture (prepared by mixing 35 ml. 0.1*M* Michaelis buffer and 15 ml. 95% ethanol) which contained 25 mg. 2,2′-dihydroxy-6,6′-dinaphthyl disulfide (DDD). After cooling 10 minutes at room temperature, the slides were rinsed briefly in water and washed 10 minutes in dilute acetic acid. The excess reagent and reaction by-product were extracted with two changes of ether for

5 minutes, after slides were passed through a graded series of alcohols. The sections were rehydrated and, after being rinsed in water, were stained exactly 2 minutes at room temperature with a freshly prepared filtered solution of the coupling reagent (either 50 mg. tetra-azotized diorthoanisidine or 50 mg. Fast Blue RR) in 50 ml. 0.067*M* Sörensen phosphate buffer. Both the time of coupling and freshness of the solution affect the staining and they must be rigidly controlled. The slides were washed in running tapwater and the cover glass mounted with glycerol.

Specificity of reaction was tested on fixed sections, wheat germ, bran, and flour, respectively. The TCA-fixed samples were washed with two changes of 80% ethanol and briefly with water. Subsequently, they were kept 1 hour at 30°C. in the oxidizing or –SH-blocking solution and, prior to reaction with DDD, washed with three changes of water. Control samples were passed through the same procedure, except that distilled water was used instead of the oxidizing or –SH-blocking solutions.

Results and Discussion

The results obtained from staining of wheat sections are shown in Figs. 2, 3, and 4.

In Fig. 2, the whole transverse section is shown. In Figs. 3 and 4, parts of the section shown in Fig. 2 are given at higher magnification. These results show clearly that the major sites of sulfhydryl groups in the wheat kernel are the germ and the aleurone layer. The sulfhydryl content of the endosperm is much less than that of the aleurone layer or the germ. In the endosperm, there is a gradual decrease of

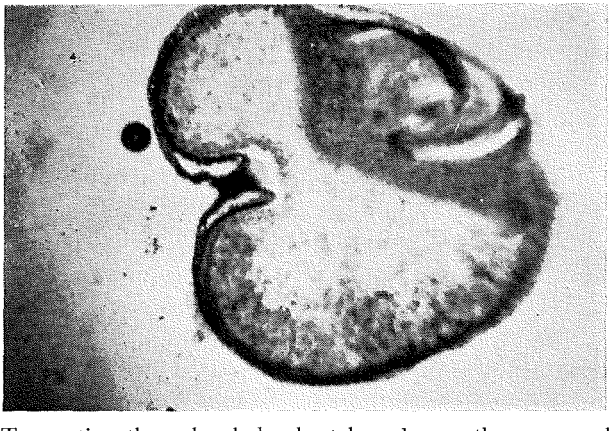

Fig. 2. Transection through whole-wheat kernel near the germ end (20×).

sulfhydryl groups from the outer layers to the central ones; the smallest concentration of –SH groups is in the central portions of the endosperm and the cheeks.

Figure 5 shows in more detail the distribution of sulfhydryl groups in the pericarp, seed coat, and adjacent layers, as well as in the aleurone layer.

Figure 6 shows that the pattern of distribution of sulfhydryl groups in the corn kernel is the same as in wheat; the major sites of sulfhydryl groups are the aleurone layer and the germ.

Data on the specificity of the reaction are given in Table I.

The reaction is inhibited to a large extent, or completely, by the

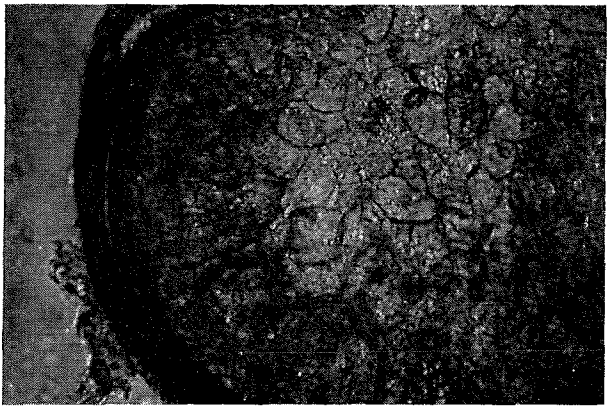

Fig. 3. Transection through wheat kernel; shows details of pericarp and adjacent tissues (50×). Detail of Fig. 2.

Fig. 4. Transection through wheat kernel shows details of embryo and layers adjacent to crease (50×). Detail of Fig. 2.

Fig. 5. Transection through pericarp and adjacent tissues in wheat (200×).

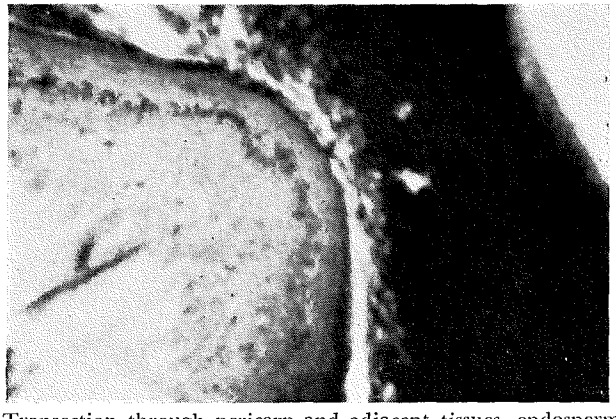

Fig. 6. Transection through pericarp and adjacent tissues, endosperm, and embryo of a corn kernel (20×).

oxidizing or blocking reagents employed. Action of the various agents used differs widely. Potassium iodosobenzoate was most effective; N-ethylmaleimide was a close second. Iodate and periodate caused high inhibition of the reaction, whereas bromate (extensively used as a flour-oxidizing agent) was least effective although it decreased staining intensity slightly.

Though the Barrnett-Seligman procedure is generally accepted as specific for –SH groups, no blocking reaction completely prevented the DDD reaction (6). During the reaction, a slightly alkaline medium (buffered at pH 8.5) was employed. It made the –SH groups more reactive (but at the same time it opened S–S linkages). Generally, animal or plant tissues that give positive tests for sulfhydryls also contain disulfides. This is because sulfhydryls and disulfides provide

TABLE I

EFFECT OF OXIDIZING AND BLOCKING REAGENTS ON THE HISTOCHEMICAL TEST OF SULFHYDRYL GROUPS IN WHEAT PRODUCTS

TREATMENT	COLOR OF TESTED SUBSTRATE			
	Sections	Germ	Bran	Flour
Control	Germ and aleurone very deep violet; endosperm violet	Very deep violet	Very deep violet	Violet
Bromate	Germ and aleurone deep violet	Deep violet	Violet	Violet
Iodate	Slight violet	Very slight violet	Very slight violet	Unstained
Periodate	Violet	Very slight violet	Very slight violet	Unstained
Iodosobenzoate	Unstained	Unstained	Unstained	Unstained
N-ethylmalemide	Very slight violet	Very slight violet	Unstained	Unstained

a truly reversible intracellular oxidation-reduction system. This may be of special importance in the histochemistry of wheat products where the predominant and relatively abundant disulfide links are liable to be broken to an appreciable extent by the DDD reagent and thus can interfere with the complete color inhibition. Even if a predominance of either sulfhydryls or disulfides is formed in certain tissues, resulting from a shift in equilibrium of the reversible redox system, prolonged incubation in DDD may affect the staining reaction or partially overcome the blockage in tests employing blocking agents.

The results of the present investigation show that the presence in wheat flour of particles originating from the aleurone and germ tissues accounts for the high levels of thiol groups in low-grade milled wheat products. The relation of sulfhydryl content to bromate requirements and the possible application of the histochemical test for visualization of protein-bound –SH groups to the quantitative estimation of sulfhydryls in wheat flour are being investigated.

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