Cereal Chemistry

The Oxidation-Reduction Enzymes of Wheat. IV. Qualitative and Quantitative Investigations of the Oxidases [1]

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ABSTRACT

Qualitative and quantitative studies of oxidases in whole wheat and five milling fractions of two hard red winter (Triumph and Bison) and spring (Lee and Selkirk) wheats were conducted. Qualitative data were obtained by polyacryamide-gel electrophoresis, and quantitative information by spectrophotometric and manometric techniques. The enzymes studied were peroxidase, cytochrome oxidase, catalase, ascorbate oxidase, indoleacetic acid oxidase, and polyphenol oxidase. Qualitatively, gel-electrophoretic patterns were similar in all varieties for peroxidase and catalase. Two catalase isoenzymes and eight peroxidase isoenzymes were detected, two of which migrated toward the cathode at pH 8.9. The activity of both enzymes was much higher in spring wheat than winter wheat. Catalase activity was four to 8 times higher in bran than flour and peroxidase was five to thirteen times higher in bran. It appeared that the cytochrome oxidase bands observed on gels were due to peroxidase. Only trace amounts of ascorbate oxidase and polyphenol oxidase were detected in spring and winter wheats. Polyphenol oxidase activity was too low to be detected on gels. Indoleacetic acid oxidase activity was detected in winter wheat only.

The quality of wheat flour depends not only on physical properties, but also on the biochemical composition of the wheat grain endosperm. The most important of the biochemical components are the proteins (1), starches (2), and lipids (3). Some attention has been directed toward the enzymes (4). The oxidative enzymes of wheat are of interest because of the possible role that they may have in modifying the properties of dough during mixing. Lipoxidase (EC 1.99.2.1) has been implicated in the oxidative polymerization of flour proteins associated with dough improvement in addition to· bleaching of carotenoid pigments (4–9). Catalase (EC 1.11.1.6), which has been studied extensively in wheat (4,10–14), can also cause a coupled oxidation of carotene (12).

Peroxidase (EC 1.11.1.7) is an abundant enzyme in most nonchlorophyllous plant tissues (15,16,17). In comparing peroxidase activities of wheat, corn, barley, and rice, Wallerstein *et al.* (18) found that wheat contained the highest activity. The same workers found a close correlation between *o*-phenylenediamine and pyrogallol oxidase activities in wheat (19,20),

which suggests that these two activities are due to a single enzyme, probably peroxidase, since o-phenylenediamine and pyrogallol react very well with peroxidase (17). Other oxidases that have been studied to a lesser extent in wheat include polyphenol oxidase (EC 1.10.3.1) (11,21), cytochrome oxidase (EC 1.9.3.1) (22), and ascorbate oxidase (EC 1.10.3.3) (21,23).

Past investigations of oxidase activity in wheat have not provided quantitative information about the isoenzyme of individual enzymes (24) present in flour. Expression of activity has been on a relative basis. The investigations on dehydrogenases of wheat by Honold et al. (25,26) have been extended to the oxidases of wheat. This paper presents the results of qualitative and quantitative studies on the identity and activity of the oxidases occurring in two winter and two spring wheats and five milling fractions (flour, break shorts, reduction shorts, bran, and red dog) of each variety.

MATERIALS AND METHODS

The oxidases of four varieties of wheat (*Triticum aestivum* L.), Triumph and Bison, hard red winter wheats, and Lee and Selkirk, hard red spring wheats, were studied. The extraction and storage of enzymes and poly-acrylamide-gel-electrophoretic separation were performed as described in a previous paper (25). The protein content of extracts was determined by the microbiuret method (27). Six oxidases were studied: peroxidase, catalase, polyphenol oxidase, ascorbate oxidase, cytochrome oxidase, and indoleacetic acid oxidase.

Qualitative Enzyme Assays

Peroxidase was assayed after electrophoresis in polyacrylamide gels with the use of guaiacol as a hydrogen donor (28). Gels were incubated in $0.02M$ guaiacol for 30 min., washed, and then immersed in $0.01M$ H_2O_2 for band development.

The *nadi* modification was used to detect "cytochrome oxidase" on gels (29). The reaction mixture consisted of 25 ml. $0.3M$ pH 7.2 potassium phosphate buffer, 1 ml. 1% alpha-naphthol in 40% ethanol, and 1 ml. 1% N,N-dimethyl-p-phenylenediamine.

Polyphenol oxidase was assayed on gels incubated in a solution containing 2 ml. $0.01M$ pyrocatechol and 4 ml. $0.25M$ pH 6.5 potassium phosphate buffer.

Catalase was assayed on gels containing 0.3% hydrolyzed starch. The gels were first immersed in 0.1% H_2O_2 for 1 min. and then incubated in 1.5% potassium iodide for negative-staining of bands; i.e., the whole gel stained a deep blue owing to the starch-iodine reaction, except where catalase destroyed H_2O_2, thus preventing the oxidation of iodide to iodine. An assay for amylase was used as a control for catalase, since starch destruction by amylase in the gels results in a negative stain that could be interpreted as catalase. After electrophoresis, the gels containing 0.3% hydrolyzed starch were incubated in a solution of 0.004% iodine and 1.5% potassium iodide in $0.2M$ acetate buffer at pH 4.8. Amylase bands were observed by negative staining.

Use of Respirometer in Enzyme Assays

The principle of differential respirometric analysis (30) as incorporated

into the Gilson differential recording respirometer was used for manometric analyses. Gas changes were monitored continuously and plotted as μliters of oxygen consumed. By assuming ideal gases, the observed oxygen uptake or evolution values were corrected to the change in volume of dry gas at standard conditions (31).

The equilibration time before the enzyme extract was dumped from the sidearm of each flask was about 10 min. The bath temperature was 30°C. Boiled (10 min.) enzyme controls were used for all respirometric analyses.

Quantitative Enzyme Assays

Peroxidase was assayed spectrophotometrically (32). The assay mixture (7 ml. total volume) consisted of 1.0 ml. 0.1M pH 6.5 potassium phosphate buffer, 3.5 ml. water, 1.0 ml. 0.02M guaiacol, 1.0 ml. 0.3% H_2O_2, and 0.5 ml. crude enzyme extract. The H_2O_2 was added last to initiate the reaction. The increase in absorbance at 470 mμ was measured at 15-sec. intervals for 75 sec. Zero time for the reaction was 15 sec. after addition of H_2O_2. Two controls were run: one with water substituted for guaiacol and the other with water substituted for H_2O_2. One unit of enzyme was defined as that amount which consumes 1 μmole of H_2O_2 per min. in a 1.0-cm. light path under the conditions of the assay.

Ascorbate oxidase was assayed respirometrically by a slight modification of the method of Dawson and Magee (33). The contents of a flask were: 0.2 ml. 10% KOH and filter paper in center well, 0.5 ml. crude enzyme extract in sidearm, and 1.25 ml. 0.2M pH 6.0 potassium phosphate buffer, 0.35 ml. 0.1% gelatin solution, 0.5 ml. water, and 0.4 ml. 0.028M ascorbic acid (250 mg. L-ascorbic acid in 50 ml. water containing 50 mg. meta-phosphoric acid) in the main chamber. One unit of enzyme was defined as that amount which converts 1 μmole of substrate to product per min. at 30°C.

Indoleacetic acid oxidase was estimated with the use of the respirometer and the assay described by Mahadevan (34). One unit of activity was defined as that amount which oxidized 1 μmole of indoleacetic acid per min., assuming that 1 mole of oxygen was consumed per mole of substrate.

Polyphenol oxidase was assayed both spectrophotometrically and respirometrically. The spectrophotometric reaction mixture (35) consisted of 1.0 ml. 0.1M pH 6.5 potassium phosphate buffer, 0.3 ml. water, 0.3 ml. crude enzyme extract, and 1.0 ml. 0.01M pyrocatechol. The increase in absorbance was measured at 390 mμ at 15-sec. intervals for 75 sec. in a 1.0-cm. light path. Zero time for the reaction was 15 sec. after addition of pyrocatechol, and the control contained water instead of pyrocatechol. The activity was expressed as the change in absorbance per min. per ml. of extract. Manometric measurements were conducted by a modification of the method described by Dawson and Magee (36). The contents of a given Warburg flask for respirometric assays were: 0.2 ml. 10% KOH and filter paper in center well, 1.5 ml. 0.01M pyrocatechol and 1.0 ml. 0.5M pH 6.5 potassium phosphate buffer in main chamber, and 0.5 ml. crude enzyme extract in sidearm. The activity was expressed in μliters O_2 consumed per min.

Preliminary experiments of assaying catalase indicated excellent agreement between spectrophotometric and manometric data (32). Respirometric

analyses were used in further work. The contents of a flask were: 0.2 ml. 10% KOH and filter paper in center well, 0.1 ml. crude enzyme extract and 1.9 ml. 0.1M pH 7.0 potassium phosphate buffer in main chamber, and 1.0 ml. 3% H_2O_2 in sidearm. Buffer was added to maintain constant volume in those cases where less enzyme extract had to be used. One unit of enzyme activity was defined as that amount which catalyzes the conversion of 1 μmole of H_2O_2 to water and oxygen per min. at 30°C.
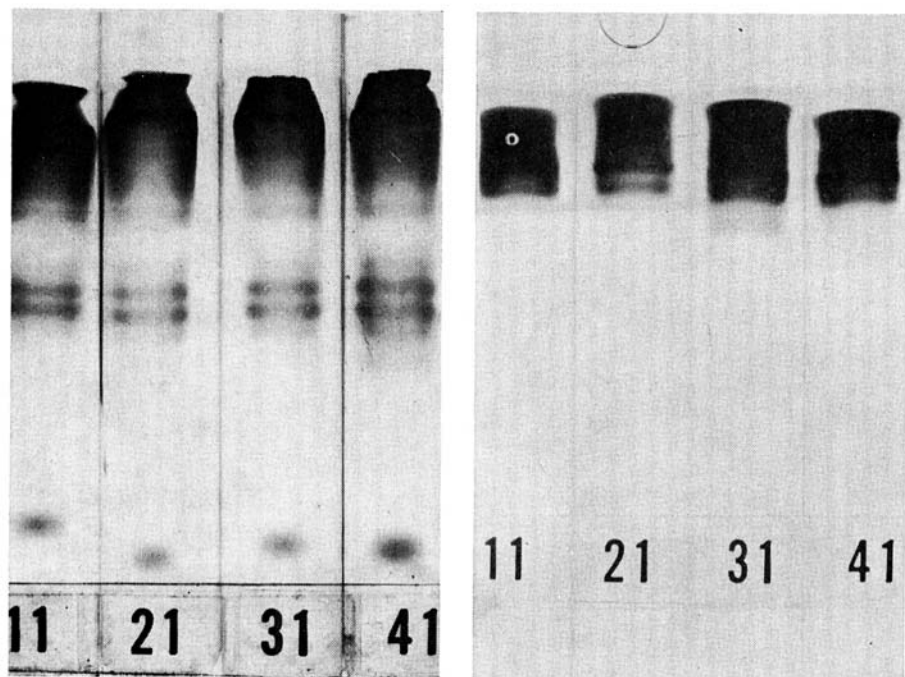

Fig. 1 (left). Polyacrylamide-gel-electrophoretic patterns or zymograms of peroxidase isoenzymes in extracts of ground whole wheat from two hard red winter wheats (Triumph-11 and Bison-21) and two hard red spring wheats (Lee-31 and Selkirk-41).

Fig. 2 (right). Polyacrylamide-gel-electrophoretic patterns or zymograms of peroxidase isoenzymes in extracts of ground whole wheat that migrate toward the cathode at pH 8.9 from two hard red winter wheats (Triumph-11 and Bison-21) and two hard red spring wheats (Lee-31 and Selkirk-41).

RESULTS AND DISCUSSION

Peroxidase

Figure 1 shows the electrophoretic patterns or zymograms of peroxidase activity in extracts of ground whole wheat for the four varieties. Extracts from break shorts, reduction shorts, and red dog contained more intense bands than the other fractions. The bands from flour extracts had the lightest intensity. Six bands plus a smear of activity near the top of the small-pore gel were detected in all samples. Some isoenzymes of peroxidase are quite basic, i.e., they migrate toward the cathode at pH 8.9. To assay for such "cathodic" peroxidases, the electrodes were reversed so that the cathode was in the lower buffer reservoir. Figure 2 shows the results of such

an experiment. In addition to considerable stain in the large-pore gel, all samples had two bands in the small-pore gel. The "cathodic" peroxidases from flour extracts were barely visible on electropherograms of winter wheat flours, but very intense bands were present in the spring wheat flours. The total of eight peroxidase bands observed by considering both the cathode and anode directions of migration is not a surprising number. Up to 16 isoenzymes have been observed on starch gels of peroxidases from other plants (37).

TABLE I

PEROXIDASE ACTIVITY IN THE EXTRACTS OF MILLING FRACTIONS FROM FOUR
WHEAT VARIETIES AS MEASURED SPECTROPHOTOMETRICALLY

FRACTION	VARIETY							
	Triumph		Bison		Lee		Selkirk	
	units[a]/ ml.	S.A.	units/ ml.	S.A.	units/ ml.	S.A.	units/ ml.	S.A.
Whole wheat	5.8	1.23	5.6	1.00	10.3	1.69	7.9	1.47
Flour	0.7	0.14	1.2	0.19	2.1	0.51	1.4	0.32
Break shorts	10.0	0.91	6.2	0.56	24.7	1.90	17.0	1.21
Reduction shorts	13.1	1.11	5.4	0.40	15.4	1.14	5.8	0.45
Bran	9.3	0.75	8.1	0.60	9.8	0.91	8.3	0.69
Red dog	6.2	0.65	3.5	0.32	17.3	1.24	12.3	0.82

[a]One unit = that amount which consumes 1 μmole of H_2O_2 per min. under the conditions of the assay. S.A. (specific activity) = units/mg. of protein.

Table I shows quantitative spectrophotometric data of peroxidase activity in the milling fractions of all varieties. The spring wheat flour and ground whole wheat fractions were substantially higher in peroxidase activity; however, this was not true in some of the other samples. It has been reported that spring wheat contains more peroxidase activity than winter wheat (11).

The quantitative values for peroxidase activity show that this enzyme is present in high levels in wheat as compared to other oxidation-reduction enzymes (26) that have been studied. Peroxidase is an unusual enzyme, as it can react with a wide variety of compounds that are capable of being hydrogen donors for the enzyme reaction (17,38). The enzyme can act directly on the amino acid residues in proteins to oxidize (39) and polymerize (40) the proteins. It has been shown that peroxidase activity increases extensively in barley and wheat upon malting (18,41) and malt is used as a source of enzymes for dough improvement[2]. The large amount of activity in the wheats used in the present experiments and the oxidative abilities of peroxidase suggest that this enzyme could play a significant role in the protein interactions that apparently take place in dough improvements during mixing. Further studies are in progress to investigate this possibility.

Cytochrome Oxidase

The staining observed by the *nadi* reaction on electropherograms does not necessarily represent a true cytochrome oxidase activity. Other enzymes, such as amine oxidase, amino acid oxidase, peroxidase, polyphenol oxidase, and indoleacetic acid oxidase, may react to give positive results. The patterns of cytochrome oxidase observed on acrylamide gels were identical with the

[2]Garner, E. Personal communication.

patterns for peroxidase in Fig. 1. Staining of gels with the *nadi* reaction for activity that migrated toward the cathode at pH 8.9 gave a pattern identical with that in Fig. 2. Since the presence of peroxidase in enzyme extracts makes it impossible to stain reliably for cytochrome oxidase (28), our results suggest that peroxidase catalyzed the *nadi* reaction, even though the presence of cytochrome oxidase in wheat has been reported (22).

Ascorbate Oxidase

Manometric data of all samples indicated that this enzyme was present in only trace levels. Milner (21) reported that the activity of ascorbate oxidase in ground wheat and bran was insignificant in comparison to that of polyphenol oxidase. Our results indicated no detectable activity in the flour fractions. These results are also in agreement with those of Carter and Pace (23), whose evidence suggested the absence of a very active ascorbate oxidase in wheat flour.

Early reports (42,43,44) demonstrated the presence of an ascorbic acid oxidase system in wheat flour and suggested that L-ascorbic acid had an effect similar to that of an oxidizing agent in dough. The improver reaction involved both an oxidation of L-ascorbic acid to dehydro-L-ascorbic acid and then a reduction of dehydro-L-ascorbic acid by reducing materials in the dough. Recent reports (23,45,46,47,48) have extended the earlier work and have conclusively shown L-ascorbic acid to be associated with improved dough characteristics. Owing to the pronounced specificity for the L-isomer of dehydro-ascorbic acid, the reaction was presumed to be enzymatic. Whether the conversion of L-ascorbic acid to dehydro-L-ascorbic acid is due to enzyme activity or due to metal ion catalysis has not been elucidated.

Indoleacetic Acid Oxidase

Indoleacetic acid oxidase activity was detected and measured on the respirometer. Activity was found only in whole-wheat fractions from the two winter varieties, Triumph and Bison, but not in the spring varieties. The enzyme had a lag phase of 2 min. in Triumph and from 30 to 60 min. in Bison. Crude enzyme extracts of the Triumph variety contained 4.9 units of enzyme activity per ml. and the Lee variety contained 3.25 units per ml. of extract. Some studies (49) have indicated that the indoleacetic acid oxidase activity of wheat is due to peroxidase. It does not appear that peroxidase catalyzed the reaction in the studies presented in this paper, since peroxidase activity was higher in the spring wheat.

Polyphenol Oxidase

The results of polyacrylamide gel analyses for polyphenol oxidase activity indicated that the activity of this enzyme was very low in all wheat samples that were analyzed. The results of the gel assays were confirmed by data of spectrophotometric and respirometric analyses shown in Table II. The activity was low in both winter and spring wheats, and, in agreement with the results of other workers (21,42), no differences in activity were found between winter and spring wheats. Brown and Goddard (22) reported no polyphenol oxidase activity in wheat germ; however, other workers (21,42) have detected polyphenol oxidase in bran in significant amounts. The polyphenol oxidase activity was found to be four to six times higher in bran

TABLE II
POLYPHENOL OXIDASE ACTIVITY IN THE EXTRACTS OF MILLING FRACTIONS FROM FOUR
WHEAT VARIETIES AS MEASURED SPECTROPHOTOMETRICALLY AND RESPIROMETRICALLY

| | VARIETY | | | | | | | |
| | Triumph | | Bison | | Lee | | Selkirk | |
FRACTION	Spec.[a]	Resp.[b]	Spec.	Resp.	Spec.	Resp.	Spec.	Resp.
Whole wheat	0.00	0.00	0.04	0.26	0.04	0.38	0.06	0.69
Flour	.02	.30	.00	0.00	.00	0.00	.00	0.00
Break shorts	.03	.38	.17	0.90	.12	0.78	.24	1.70
Reduction shorts	.07	.80	.20	1.35	.22	1.70	.36	2.24
Bran	.00	.59	.32	1.93	.16	1.22	.28	1.66
Red dog	0.04	0.39	0.07	0.96	0.08	1.34	0.20	2.07

[a] Spec. = Change in absorbance per min. per ml. of enzyme extract at 390 mμ as measured with the spectrophotometer.
[b] Resp. = μl. O_2 uptake per min. per ml. of enzyme extract as measured with the Gilson Differential Recording Respirometer.

than in whole wheat on a relative basis (11,21,42). These ratios are generally the same as those obtained in the studies presented in this paper. Because of the portion of the wheat kernel that is bran, this suggested that most of the polyphenol oxidase from wheat is in the bran. The results of these studies on polyphenol oxidase suggest that the amount of this enzyme is negligible in wheat flour. This would depend, however, upon the way the wheat is milled.

Catalase

Polyacrylamide-gel assays of catalase activity revealed two bands of catalase close together that migrated about one-fourth as far as the bromphenol blue marker dye. The qualitative pattern was the same for the milling fractions of all wheats. The negative-staining was very light in gels containing flour extracts.

TABLE III
CATALASE ACTIVITY IN THE EXTRACTS OF MILLING FRACTIONS FROM FOUR WHEAT
VARIETIES AS MEASURED RESPIROMETRICALLY

| | VARIETY | | | | | | | |
| | Triumph | | Bison | | Lee | | Selkirk | |
FRACTION	units[a]/ ml.	S.A.	units/ ml.	S.A.	units/ ml.	S.A.	units/ ml.	S.A.
Whole wheat	29	6.2	50	8.9	109	17.9	72	13.3
Flour	7	1.4	12	1.9	67	16.0	37	8.4
Break shorts	137	12.5	180	16.4	879	67.6	774	55.3
Reduction shorts	123	10.4	217	16.0	950	70.4	550	42.6
Bran	134	10.7	219	16.1	573	53.2	393	32.8
Red dog	76	8.0	124	11.5	912	65.0	356	23.7

[a] One unit = that amount which converts 1 μmole of hydrogen peroxide to water and oxygen per min. at 30°C. S.A. (specific activity) = units/mg. of protein.

Manometric data obtained on the respirometer for all samples are presented in Table III. The levels of activity were substantially higher in the spring wheats. The average level of activity in the spring wheat flours was about five times as high as the average for the winter wheats. Other workers (4,10,14) have reported that the activity was lowest in the central part of the endosperm. This is in agreement with the results observed in these

experiments. On a specific basis, catalase was most active in the shorts fractions. The bran of winter wheat contained about the same activity as the winter wheat shorts, whereas the spring wheat bran was somewhat lower in catalase than the spring wheat shorts. Hawthorn and Todd (12) reported that bran contains twice the catalase activity of flour as compared to the results in Table III, which indicate four to eight times more activity in the bran. Thus, small amounts of bran contamination in flour would greatly affect the catalase content of the flour.

Catalase catalyzes one of the fastest biological reactions known, the conversion of H_2O_2 to water and oxygen. It has long been thought that this is the only reaction which involves catalase. However, other compounds (17) can act as hydrogen donors (substances that are oxidized in peroxidase- or catalase-catalyzed oxidations) for the catalase-hydrogen peroxide complex. Free tyrosine was one of the substances that served as donors in model studies (17). This raises the question of whether or not amino acid residues in proteins could act as hydrogen donors for catalase or peroxidase. If catalase or peroxidase could utilize as donors the amino acid residues bound into the peptide chain of proteins, the high activity of these enzymes in wheats may be able to affect protein polymerization in doughmaking.

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