Test Paper for Detecting Peroxidase

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A procedure is described for preparing test papers for detecting peroxidase. Qualitative tests for peroxidase are made by merely moistening the paper with the solution to be tested. Stability of the paper is greatly increased by storage at very low humidity under refrigeration.

AFTER HARVEST and during processing of many agricultural crops, two types of undesirable changes in the plant material may occur. Desirable substances—for example, vitamins—may be destroyed, or substances may be produced that cause objectionable changes in color or flavor. Both types of change are known to be catalyzed by naturally occurring enzymes. To prevent or to reduce these changes, the fresh tissue is usually subjected to either steam or hot-water blanching. By this means, the enzymes can be inactivated quickly, preventing their catalytic action. Excessive blanching may be objectionable, however, from the standpoint of economics or product quality. The best product quality—based on color, texture, and flavor, in vegetables for example—is produced by the minimum blanching required to destroy enzymes.

Enzymes vary in heat lability. They also vary in ease of detection or measurement. One of the most heat-stable enzymes, peroxidase, is also one of the easiest to detect or measure. These factors, coupled with its almost universal presence in plant material, make peroxidase a very convenient enzyme for use in evaluating the extent of blanching in many products. The literature contains many reports (*1–3, 5, 7*) of both qualitative and quantitative methods for peroxidase determination. However, the method described here is believed to be more simple and less subject to errors in manipulation.

A peroxidase is an enzyme that catalyzes the oxidation of a substrate with aid of a peroxide and is of low specificity with regard to its substrate. The peroxide most often used in both qualitative and quantitative procedures is hydrogen peroxide.

As enzyme measurements depend on rates of the reactions, the concentration of substrate and hydrogen peroxide must be controlled by tedious measurements of reagents of known concentration (*7*). Whereas the research chemist is accustomed to these details, some control chemists, as well as food technologists, may not appreciate the need for or have the facilities to control the enzyme tests properly. Hence, food processors have long desired a simpler test for control work.

A satisfactory substrate and a peroxide for the enzyme action are basic needs. Peroxidases have a low order of substrate specificity; therefore the selection of a substrate was simple. However, most organic peroxides are very inefficient for peroxidase action (*8*). Urea peroxide functions as efficiently as hydrogen peroxide for both catalase and peroxidase action, also it is a solid, and is reasonably stable at room temperature.

Experimental

Procedure. Fresh, 1% alcoholic solutions of urea peroxide and of *o*-tolidine are prepared. In all cases, the reagent was used within 1 or 2 minutes after preparation; its storage stability was not studied. Immediately before use, equal volumes of these solutions are mixed to give a solution that contains 0.5% of each substance. The filter papers are immersed just long enough to become completely saturated, then pressed firmly to remove most of the excess solution, and air dried.

Under laboratory conditions, 50 to 100 papers are prepared at a time. The excess solution is removed by placing the stack of papers between stainless steel plates and applying pressure by means of a C-clamp held in a vise. To facilitate air drying, the papers are perforated near the edge with about a $^3/_8$-inch hole prior to processing, and after pressing they are strung on a string and spaced 1 or 2 inches apart. The drying, which takes 20 to 30 minutes, is carried out preferably in a low-humidity room under low light intensity. The prepared papers are stored over anhydrous calcium chloride under refrigeration.

Test Paper Stability. The useful life of the paper was assumed to be determined by the stability of the peroxide deposited on it. Hence, a study was made of the peroxide contents of some papers stored over anhydrous calcium chloride in fruit jars for different intervals at 40°, 75°, and 100° F. The papers were prepared from 9-cm. Whatman No. 50 filter papers with the solution described above.

The classical method of titrating peroxide with permanganate was unsatisfactory, because the *o*-tolidine present interfered. Therefore, the method used was essentially that of Morris, Weast, and Lineweaver (*6*). A whole filter paper was placed in the 2*N* sulfuric acid for a few minutes to dissolve the peroxide, and the determination was carried out without removing the paper. The peroxide values in Table I are averages of four determinations.

Table I shows that temperature is a very important factor governing the peroxide stability. Papers held at 100° F. lost one half of their peroxide in 18 days. At 75° F., three fourths of their peroxide was retained for 55 days, but had dropped to 30% of the original value after 3 months. No significant loss occurred in 3 months on papers stored at 40° F.

Discussion

Test paper eliminates the need for a reaction vessel, pipets, or burets. The only requirement in making a qualitative test for peroxidase activity is that the

Table I. Peroxide Stability on Test Papers[a] Stored at Various Temperatures

Days Stored	Storage Temperature, °F. 40	75	100
7	108	80	76
18	103	92	49
35	107	73	23
55	122	73	..
90	97	29	..

[a] Initially, papers contained equivalent of 0.190 mg. H_2O_2. Values shown are percentages of original content.

paper be moistened with the sample to be tested. This can be done in two ways: A drop of an extract can be added to the paper or the paper can be touched with the moist surface of a solid piece of the test material. The choice of method depends on the information desired. The result obtained with a drop of extract reflects the average peroxidase activity of all the samples used in preparing the extract, but by touching the paper with the moist surface of a piece of solid material, variations in the peroxidase activity of different areas of the piece can be observed. For example, a blue circle appeared on paper that was touched by a freshly cut cross section of blanched alfalfa root. The blue circle coincided with the phloem of the root, which showed that this part of the root still contained active enzymes whereas the center (xylem) was peroxidase-free. If a significant amount of peroxidase activity is present, a positive reaction occurs in less than a minute.

The use of the paper to indicate peroxidase-active portions of food material may be valuable to food processors. The method is unique as an enzyme method in being able to measure activity in situ.

The specificity of prepared papers for peroxidase is the same as using the particular substrate in conventional procedures. For example, papers prepared with *o*-tolidine will give a slight positive reaction with active iron preparations such as hemoglobin. Catalase will not give a positive reaction.

Some of the variables that have been studied are different substrates, ratio of substrate to urea peroxide, optimum amounts of these substances on the paper, quality of filter paper, and different solvents for the reagents.

Test papers have been prepared with benzidine, *p*-phenylenediamine, pyrogallic acid, and *o*-tolidine as substrates. *o*-Tolidine is preferred because of the vivid blue color of the peroxidase-oxidized product and the better stability of the prepared paper.

Papers were prepared in which the ratio of urea peroxide to substrate varied from 10:1 to 1:10. Within the range studied a ratio of 1 to 1 is satisfactory. Too large an excess of peroxide caused the papers to turn blue on drying, whereas an inadequate amount decreased the sensitivity of the paper.

With urea peroxide and *o*-tolidine in the ratio of 1 to 1, the concentrations of these substances in the solution used to prepare the paper were varied from 0.1 to 1.0%. The highest concentrations were unsatisfactory, in that the prepared papers showed undesirable browning on short storage. The lowest concentrations gave paper of low sensitivity. Papers prepared from a solution containing 0.5% of each reagent were satisfactory.

Several kinds of filter paper, from 7 to 11 cm., were used and found satisfactory—i.e., Whatman's Nos. 1 and 50, Carl Schleicher and Schuell Co. Nos. 576, 595, and 597. Heavy paper, such as Whatman's No. 3, was less satisfactory because of discoloration on drying.

Absolute ethyl alcohol, 95% ethyl alcohol, and 99% isopropyl alcohol have been used successfully as solvents for urea peroxide and the substrate. Solvents such as ethyl ether and acetone were not used because of the hazardous nature of peroxides of these compounds.

A detailed comparison of the sensitivity of the test paper procedure with other published methods has been made on a series of vegetables. The results of this study (*4*) showed that the test paper is as sensitive as the Masure and Campbell (*3*) qualitative test (often referred to as the U.S.D.A. method), which is the method of choice of many food processors.

The effects of light and humidity on paper stability have not been studied in detail but are known to be important. Papers exposed to ordinary room light and uncontrolled relative humidity lost much of their sensitivity to peroxidase in a few hours. Desiccated papers stored in the dark at room temperature retain good quality for a week or more, and, if refrigerated, they remain serviceable for several months.

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PRE-FERMENTS IN BREADMAKING

Organic Acids and Esters Produced in Pre-ferments

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THE FLAVOR of fresh bread (*7*), originating largely through fermentation (*3*) and baking (*2–4*), has universal appeal. The usual baking procedure is known as the sponge process, which consists of allowing the yeast to ferment sugar in the presence of approximately 60% of the total water and flour. After 3 to 5 hours, the sponge is mixed with the remaining ingredients to produce a dough which is then divided, molded, proofed, and baked in the customary manner.

Recently, two breadmaking processes have attracted widespread interest: the pre-ferment process (*9, 17–20*) and the continuous process (*1, 5, 12*). Neither requires the mixing of a sponge and both depend on a liquid ferment consisting mainly of yeast, water, sugar, and an inorganic or milk buffer to produce the flavor constituents normally originating in the sponge. Bacteria present in the ingredients may also contribute to the flavor through fermentation. Although the industrial application of the pre-ferment and continuous baking processes is not widespread, probably some type of pre-ferment (*6*) will eventually be used in the baking industry in conjunction with a continuous mixing process. Possibly, the pre-ferment may be supplanted by a synthetic mixture of the chemical components which normally are produced in the sponge or in pre-ferment solutions.

The effect of fermentation time on the chemical constituents of pre-ferments used in breadmaking has been reported