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ENZYME ASSAY

Quantitative Determination of Peroxidase in Sweet Corn

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A procedure is described for the quantitative determination of peroxidase in whole-kernel sweet corn. The method involves the colorimetric determination of the color formed when the enzyme oxidizes orthophenylenediamine in the presence of hydrogen peroxide. Starch is removed from a buffered extract of corn by alcohol precipitation and centrifugation. The absorption curve for the oxidized dye shows a maximum absorbance at 430 mμ under the conditions used in this assay. The color formed from the peroxidase-catalyzed oxidation of the orthophenylenediamine follows the Beer-Lambert law if a correction term is applied for the nonenzymatic oxidation of the dye. The assay is independent of the sample weight used for color development. Enzyme activity was determined in corn which had been steam blanched for various periods from 0 to 8 minutes. Enzyme activity decreased with increasing blanch time.

DATA—mostly qualitative—on the destruction of peroxidase in many vegetables may be found in the literature (*2, 4, 8*). No thermal destruction data for sweet corn peroxidase are available, as no satisfactory quantitative procedure has been worked out for its determination. This study was undertaken in an effort to develop such a method.

Although the most resistant microorganisms may be destroyed in a matter of seconds as processing temperatures approach 300° F. (*3*), certain enzymes are not completely and irreversibly inactivated by such a thermal process (*5*). Peroxidase has been particularly noted for having more heat resistance than bacterial spores at high temperatures. Storage of processed foods containing this enzyme may lead to quality deterioration.

The destruction of peroxidase in processed foods has been studied to determine the relationship between this enzyme and off-flavor development during storage. A "viny" off-flavor developed in canned peas processed under high-temperature, short-time conditions when peroxidase was not completely and irreversibly destroyed (*5*). Labbee and Esselen (*7*) reported that off-flavors developed in fresh-pack pickles when peroxidase was not completely destroyed during the pasteurizing operation. Dietrich and coworkers (*7*) reported that blanched frozen peas containing active peroxidase received flavor scores lower than those for samples containing no enzyme after storage at −10° F. Their finding agreed with those of Joslyn (*6*), in which he found that peroxidase activity paralleled the formation of off-flavors.

Most of the common methods of estimating peroxidase activity are colorimetric. Guyer and Holmquist (*5*) and Farkas, Goldblith, and Proctor (*4*) used variations of the guaiacol oxidation procedure. However, this procedure cannot be used for determining small concentrations of peroxidase in sweet corn, as the large amount of corn required to give sufficient enzyme for adequate color development results in starch turbidity which prevents direct reading in the spectrophotometer.

Wallerstein and associates (*10*) developed a fluorophotometric procedure for the quantitative determination of peroxidase in potato tubers using orthophenylenediamine as substrate. Reddi, Esselen, and Fellers (*9*) determined the amount of peroxidase in apple tissue also using *o*-phenylenediamine as substrate. They stopped the reaction after 5 minutes by adding 2 ml. of saturated solution of sodium bisulfite to 15 ml. of reaction mixture. The color was extracted with ethyl acetate and its absorbance measured in an Evelyn photoelectric colorimeter using filter No. 420.

Because the product formed by the peroxidase-catalyzed oxidation of *o*-phenylenediamine was found to be relatively stable, this method was believed to be usable for the quantitative determination of small amounts of peroxidase in sweet corn.

Recommended Procedure

Approximately 4 grams of fresh whole-kernel corn is made to a total volume of 250 ml. with pH 6.5 phosphate-citrate buffer and is blended for 3 minutes in an Osterizer. The buffer is prepared by combining 14.2 parts of 0.2*M* disodium hydrogen phosphate and 5.8 parts of 0.1*M* citric acid. Ten milliliters of this extract is diluted to 250 ml. with buffer. A 25-ml. aliquot of the blended and diluted sample is transferred to a 250-ml. centrifuge bottle. Another 25-ml. aliquot of each sample is taken for a colorimeter blank reading. The sample must be mixed thoroughly before the aliquot is taken, because much of the enzyme is located in solid particles which tend to settle on standing. The samples are placed in a constant temperature bath at 25° C. for 30 minutes.

To the sample, in which the color is to be formed, the following reagents are added: 1 ml. of 1% *o*-phenylenediamine (in 95% ethyl alcohol; fresh every 4 hours) and 1 ml. of 0.3% hydrogen peroxide (in distilled water). The reaction is allowed to proceed for 5 minutes,

at which time it is stopped by adding 2 ml. of saturated sodium bisulfite. The reagent blank for each sample is prepared by adding the dye, followed by the sulfite, and then the hydrogen peroxide. The enzyme is inhibited by the sulfite so that it is inactive when the hydrogen peroxide is added.

The starch in the sample and the blank is flocculated by adding 25 ml. of 95% ethyl alcohol. The starch suspension must be swirled continuously during addition of the alcohol, so that good flocculation occurs.

The samples are then centrifuged at approximately 3000 r.p.m. for 5 minutes. The clear supernatant is decanted into a colorimeter tube and its absorbance recorded at 430 mμ. The colorimeter is set at 100% transmittance with the corresponding blank for each sample.

If 4 grams of fresh corn are weighed and diluted as described and an absorbance reading of 0.5 is obtained, the results may be calculated as follows:

$$\frac{0.5}{\frac{4}{250} \times \frac{10}{250} \times 25} = \frac{31 \text{ absorbance units}}{\text{gram}}$$

When larger samples are taken and fewer dilutions are made, absorbance-units-per-gram values can be calculated in a similar fashion.

Experimental

Optimum pH. A sample of whole-kernel corn was blended in distilled water. Aliquots of this extract were diluted 1 to 10 with phosphate-citrate buffers of pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5. Aliquots of each of the buffered diluents were taken for color development. A plot of pH *vs.* absorbance showed a bell shaped curve with a peak at pH 6.5. This pH was used as the pH of maximum activity for sweet corn peroxidase oxidation of orthophenylenediamine.

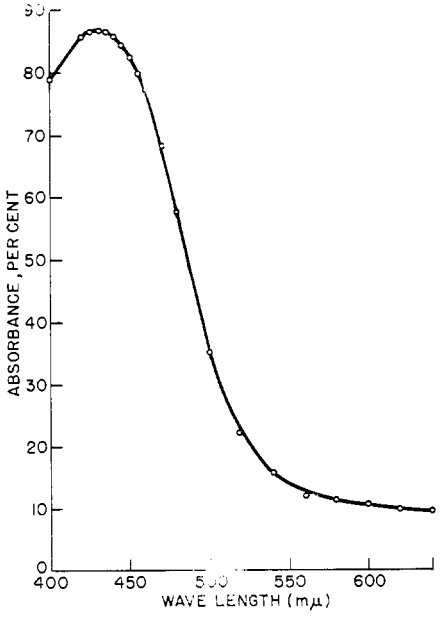

Figure 1. Absorption curve for peroxidase-oxidized o-phenylenediamine

Absorption Curve. The wave length of maximum absorption was ascertained for peroxidase-oxidized orthophenylenediamine under the conditions used in this assay. The absorption of light over the range of 400 to 700 mμ for the color developed from a typical sample of whole-kernel corn is given in Figure 1. This curve has a peak at 430 mμ. The peak is broad so that wave lengths between 420 and 440 mμ may be used with little loss of sensitivity.

Conformity to Beer-Lambert Law. The dependence of the color intensity on the quantity of enzyme taken for the assay was examined. Approximately 4 grams of whole-kernel corn were blended in 100 ml. of buffer and then diluted to 250 ml. with buffer. A portion was further diluted 1 to 25 with buffer. Aliquots of the final dilution were taken and made up to 25 ml. with buffer for color development.

Table I contains the results of this test. Concentration units are expressed as milliliters of diluted extract used. These data represent a straight line having a y-intercept of 0.030 for the zero corn sample and a slope of 0.028 absorbance units per ml. of extract. There is apparently a nonenzymatic oxidation of the dye which is accelerated by the presence of hydrogen peroxide. Addition of saturated sulfite solution to stop the enzymatic reaction obviously changes the oxidation-reduction potential of the solution in such a manner that the dye is no longer oxidized. Data to support this conclusion are presented in Table II. The absorbance readings are for three samples containing varying amounts of enzyme. After addition of sulfite, the absorbance of the solution was constant for at least 2 hours.

Whenever a fresh dye solution was prepared, a determination was made on a sample containing no enzyme (zero corn) and the small absorbance reading obtained was subtracted from all other readings obtained with this dye solution. In this way, the assay follows the Beer-Lambert law, and more accurate calculations of enzyme content are possible.

Reaction Time. A sample of unblanched corn was blended in 100 ml. of buffer and diluted to 2000 ml. Aliquots of this extract were used to determine the dependence of absorbance on the time of reaction. The oxidation of the dye in the presence of the enzyme extract was allowed to proceed for times varying from 0.5 minute to 10 minutes.

The absorbance values increased from 0.020 at 0.5 minute to 0.37 after 10-minute reaction time. The rate of increase became progressively lower as reaction time was lengthened, showing that timing of the reaction must be exact and that the same reaction time should be used in comparing two or more samples. The authors chose a 5-minute reaction time, which gave a sufficiently high absorbance for good sensitivity but was not long enough to involve a danger of reagent depletion.

Weight of Sample and Reproducibility. Whole-kernel sweet corn, in which part of the peroxidase was inactivated by heating for 1 minute in steam at 210° F., was used to determine the effect of sample weight. Aliquots of from 1 to 9 grams were weighed, diluted to 100 ml. with pH 6.5 buffer, and blended. The color was developed in 20 ml. of the extract.

The results of this experiment are

Table I. Amount of Color Formed in Samples Containing Varying Amounts of Peroxidase

Concn. Units	Absorbance	Corrected Absorbance	Absorbance/ Concn. Unit
25	0.752	0.722	0.029
20	0.630	0.600	0.030
15	0.478	0.448	0.030
12	0.392	0.362	0.030
10	0.355	0.325	0.032
8	0.311	0.281	0.036
6	0.223	0.193	0.032
4	0.160	0.130	0.033
2	0.092	0.062	0.030
0	0.030	0.000	...

Table II. Stability of Color Formed by Peroxidase-Catalyzed Oxidation of o-Phenylenediamine after 2-Minute Reaction Time

Time after Reaction Stopped by Addition of Sulfite, Hr.	Sample No. 1	2	3
	Absorbance		
0	0.197	0.230	0.283
0.5	0.197	0.230	0.283
1.0	0.197	0.230	0.283
2.0	0.197	0.230	0.283
3.0	0.195	0.230	0.278
4.0	0.188	0.230	0.270
5.0	0.183	0.222	0.262
6.0	0.163	0.208	0.245

Table III. Enzyme Activity in Different Weight Aliquots from One Batch of Heated Corn

Aliquot Wt., G.	Corrected Absorbance	Corrected Absorbance Units/G.
8.97	1.18	0.53
8.13	1.06	0.52
6.99	1.04	0.60
5.99	0.82	0.55
5.00	0.88	0.70
4.12	0.705	0.69
2.99	0.585	0.78
2.08	0.308	0.59
1.01	0.188	0.74

Table IV. Retention of Original Peroxidase Activity in Blanched, Cut Sweet Corn

Blanch Time, Min.	Absorbance Units/G.
0	20.5
1	0.780
2	0.600
4	0.495
6	0.399
8	0.287

given in Table III. The enzyme concentration found was independent of the weight of sample taken for color development; therefore, there are no heat-stable compounds in whole-kernel corn that may lead to an error in the assay.

The data in Table III also indicated the reproducibility of the assay when the enzyme content was determined for several samples taken from a batch of heated corn. The enzyme concentration values ranged from a minimum of 0.52 to a maximum of 0.78. This difference constitutes a variation of 33%. This degree of accuracy is also shown by the data in Table I. Here, the variation is approximately 20% between the maximum and minimum absorbance per concentration unit values. The data in Table I were obtained by taking aliquots of a single dilution of unheated corn. Therefore, the variation should be attributed to manipulations during the assay and not to differences in the enzyme content of heated corn. However, an enzyme assay having a variation of about 30% is believed adequate for most food analyses—particularly when small concentrations such as 1% of original enzyme content are being determined.

Application of Assay to Blanched Corn. Freshly harvested sweet corn was used in applying the enzyme assay to sweet corn blanched for various times to 8 minutes. The corn was husked, removed from the cob, washed in a rod-reel washer, and blanched in steam at 210° F. The corn was air-cooled immediately after blanching.

Appropriate weights of samples were blended for color development. The enzyme concentrations were determined and the results expressed as absorbance units per gram of corn. The results in Table IV showed that increasing blanch times gave decreasing enzyme activity.

Acknowledgment

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FOOD IRRADIATION

Determination of Micro Quantities of Methyl Mercaptan in Gamma-Irradiated Meat

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A sensitive method for determining micro amounts of methyl mercaptan produced in gamma-irradiated meat has been developed which is based on the reaction with *N,N*-dimethyl-*p*-phenylenediamine. A soluble red-colored complex is formed when methyl mercaptan is reacted with the amine solution. Hydrogen sulfide is removed by the formation of an insoluble complex in a trapping solution containing mercuric acetate. The odorous vapors from irradiated meat are carried with a stream of nitrogen into a trapping tube containing the mercuric acetate. The color is developed by adding a mixture of the acid amine solution and Reissner's solution. The intensity of the soluble red-colored complex developed is measured in a photoelectric spectrophotometer, at a wave length of 500 mμ. The method can be used for the quantitative estimation of 5 to 110 γ of methyl mercaptan.

PRELIMINARY STUDIES by Batzer and Doty (*2*) to elucidate the nature of undesirable odorous compounds developed by gamma irradiation of fresh meat indicated that, among other things, the sulfur-containing compounds—hydrogen sulfide and methyl mercaptan—developed during this process of cold sterilization. Herk and coworkers (*4*), using gas partition chromatography and mass spectrometry, isolated and identified several simple sulfides and disulfides from gamma-irradiated meat.

Marbach and Doty (*5*) described a method for determining micro quantities of hydrogen sulfide from irradiated meat. The classical reaction between *N,N*-dimethyl-*p*-phenylenediamine and hydrogen sulfide in the presence of hydrochloric acid and an oxidizing agent, which resulted in the formation of methylene blue as first proposed by Emil Fischer in 1883 (*3*), was the basis for their improved quantitative method. The use of a saturated cadmium hydroxide–sodium hydroxide solution at a pH of 13 was an effective trapping agent for