

VITAMIN C IN VEGETABLES

IV. ASCORBIC ACID OXIDASE*

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The oxidation of ascorbic acid to dehydroascorbic acid may be catalyzed by copper (1, 8), by other metals (11) and metallic compounds, by norit charcoal (5), and by an enzyme or enzyme complex (12, 13). The dehydroascorbic acid has an antiscorbutic activity similar to that of ascorbic acid (6) and may be reduced to the latter by the use of H_2S and other reductants (14). The existence of ascorbic acid oxidase in cabbage leaves was shown by Szent-Györgyi (12) in 1931. Tauber, Kleiner, and Mishkind (13) prepared from Hubbard squash a highly active enzyme which is claimed to be specific for ascorbic acid inasmuch as it does not catalyze the oxidation of cysteine, tyrosine, glutathione, and phenols. The ascorbic acid may further change into a compound which cannot be reduced to ascorbic acid and which is inactive as an antiscorbutic agent. According to Borsook and Jeffreys (3), this compound, yet unidentified, is not an oxidation product.

The present paper deals with the enzyme complex participating in the oxidation of ascorbic acid in spinach, beans, peas, pumpkin, turnips, and cabbage. Results of experiments dealing with the thermal inactivation of the enzyme are presented and the practical importance of the enzyme from the standpoint of nutrition and food preservation is discussed.

EXPERIMENTAL

During the extraction of minced vegetables with water much of the ascorbic acid present is oxidized into dehydroascorbic acid (4)

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which is physiologically active but is not detected by the indophenol dye. In water extracts of vegetables like cabbage, sweet corn, parsnips, pumpkin, and squash, which contain a very active enzyme, the ascorbic acid is entirely oxidized during the extraction period of 10 to 15 minutes, but can be completely recovered by immediate treatment with H_2S followed by elimination of the excess H_2S by CO_2 . In the case of other vegetables, such as string beans and Lima beans, peas, Swiss chard, carrots, and especially spinach, the enzymic oxidation is much slower and a considerable portion of the ascorbic acid may be extracted with water in the original form. Heating for a period sufficient to inactivate the oxidase has a beneficial effect on the preservation of ascorbic acid in vegetables, but it does not prevent losses caused by other catalysts. The extent of loss by non-enzymic catalysis varies considerably in different vegetables.

Our main interest being in the practical significance of the enzyme action rather than in a study of the enzyme itself, all the work reported here was performed on vegetable extracts which were used without further attempts at purification. It was of considerable importance to establish the extent of heating needed for the inactivation of the ascorbic acid oxidase in vegetables. To exclude the great differences in the rate of heat penetration, this was done under uniform conditions. The vegetable was ground with an equal weight of water and 5 cc. of this mixture were immersed in boiling water for a definite time interval. After cooling, 1 cc. of a solution containing 0.5 mg. of ascorbic acid was added and the mixture kept at 30° for 3 hours. Changes in the ascorbic acid content were followed by titrating the mixture by the usual method with the indophenol dye. The results obtained are shown in Fig. 1. For reasons to be discussed later, this figure also shows the relative catalase activity of the similarly heated samples as determined at pH 7 by the usual method, the residual hydrogen peroxide being estimated after 1 hour.

It appears from Fig. 1 that the ascorbic acid oxidase was completely inactivated in all cases in 1 minute, and no further changes in the enzyme activity were caused by prolonged heating. The loss caused by non-enzymic catalysis is considerable during the 3 hour period, and different blanks must be run for every period of heating. It was found that the enzymic catalysis of the oxida-

tion is rather slow in spinach and that the non-enzymic catalysis of the oxidation was more rapid than in any of the other vegetables studied. This fact might be attributed to the high iron and copper content of spinach. In Fig. 2 the results obtained with a spinach extract are compared with those obtained with a cabbage extract. The oxidation caused solely by the enzyme action may be calculated by subtracting the value for the heated from that of the

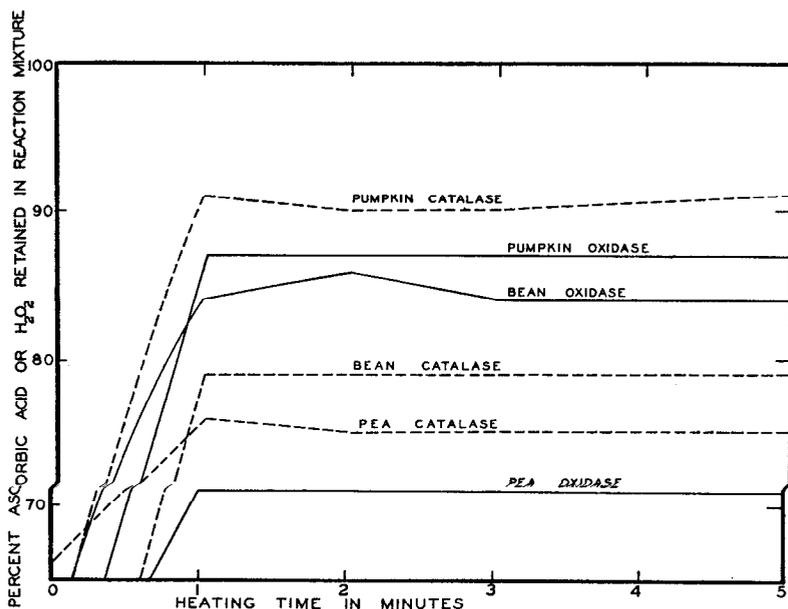


FIG. 1. Inactivation of ascorbic acid oxidase and catalase in vegetable extracts at 100°.

unheated extracts. It is apparent that the enzymic oxidation in spinach is only a fraction of that observed in the cabbage extract.

In spinach and beans cooked in boiling water for 1 minute the ascorbic acid oxidase was completely inactivated. Peas require from 30 to 60 seconds of heating in boiling water or steam to obtain a blanch sufficient to preserve desirable characteristics when the peas are to be frozen. The most desirable duration of scalding is usually established by a determination of the catalase in the vegetable. For best results no catalase activity should be present in an extract of the blanched vegetable. It was of importance

to know, therefore, the relation between the rate of heat inactivation of catalase and ascorbic acid oxidase.

It was shown in Fig 1 that there is a good correlation between the inactivation of ascorbic acid oxidase and catalase in extracts of pumpkins, beans, and peas. In Table I similar information is presented on whole peas heated for different lengths of time. In all cases, both the ascorbic acid oxidase and the catalase were

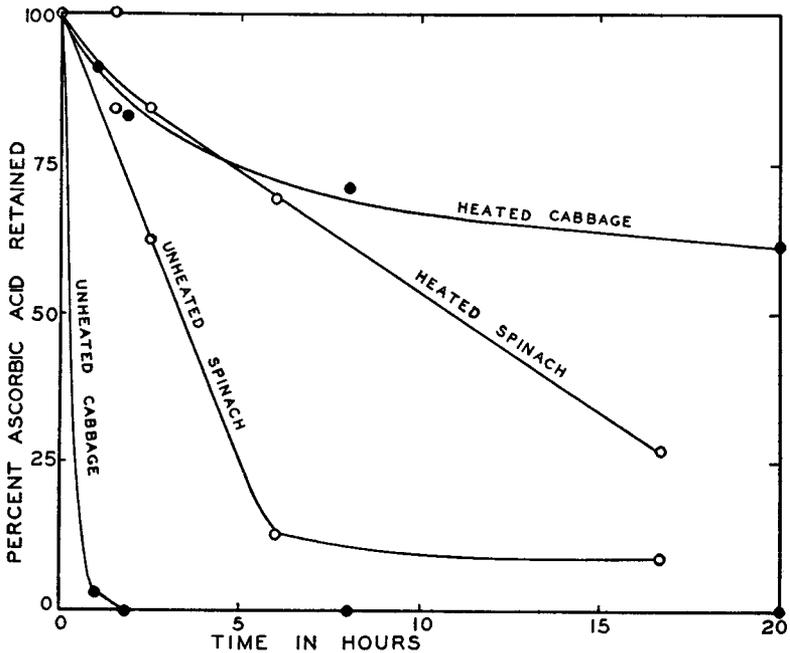


FIG. 2. Oxidation of ascorbic acid in raw and heated cabbage and spinach extracts.

completely inactivated by 1 minute of heating, while half a minute of heating gave a complete inactivation of the ascorbic acid oxidase in the steam-blanching peas only. The heating required to inactivate the catalase also inactivates the ascorbic acid oxidase.

To test the influence of the inactivation of ascorbic acid oxidase on the preservation of ascorbic acid, vined (machine-shelled) Thomas Laxton peas, blanched in steam for 30, 60, 120, and 300

seconds, were quick-frozen in a Birds Eye multiplate freezer and stored at -7° for 2 months. The ascorbic acid content of the samples was then determined by a modified chemical method (10). The results of these determinations are given in Fig. 3. In agreement with the results of enzyme determinations, the maximum retention of ascorbic acid was reached by 1 minute of heating, which was sufficient for the complete inactivation of both ascorbic acid oxidase and catalase. Attempts to regenerate the lost ascorbic acid by treatment with H_2S failed except in the unblanched sample in which case there was a nominal increase.

TABLE I
*Heat Inactivation of Ascorbic Acid Oxidase and Catalase in Thomas
Laxton Peas*

Duration and temperature of blanching	Relative enzyme activity	
	Ascorbic acid oxidase (per cent of added ascorbic acid lost in 3 hrs. at 30°)	Catalase (per cent of added H_2O_2 lost in 1 hr. at 30° , pH 6.8)
No treatment.....	54	74
0.5 min. at 82°	23	35
2 " " ".....	17	39
5 " " ".....	17	
0.5 " with steam.....	13	73
1 " " ".....	14	37
2 " " ".....	12	36
5 " " ".....	14	42

Dehydroascorbic acid may be completely reduced to ascorbic acid if the treatment is performed without delay. After a certain time, however, the length of which depends on the material and on the experimental conditions, the dehydroascorbic acid is further transformed into a compound or compounds which are biologically inactive and cannot be reduced to ascorbic acid in the usual way. In an experiment on cabbage, the results of which are presented in Table II, the different steps of decomposition of ascorbic acid in the presence and absence of the oxidase are shown. An aqueous extract of 20 gm. of cabbage per 100 cc. was prepared. A part of this extract was heated to 100° for 3 minutes and cooled. 5 mg.

of ascorbic acid solution were added to 50 cc. portions of the heated and unheated extracts. At given time intervals an aliquot of this mixture was titrated with the indophenol dye. The "regeneration" was performed by bubbling H_2S through the solution for 10 minutes, letting the sample stand in the corked test-tube for 1 hour, and bubbling CO_2 through the solution for 2 hours or until the test, as proposed by Johnson and Zilva (7), for H_2S was

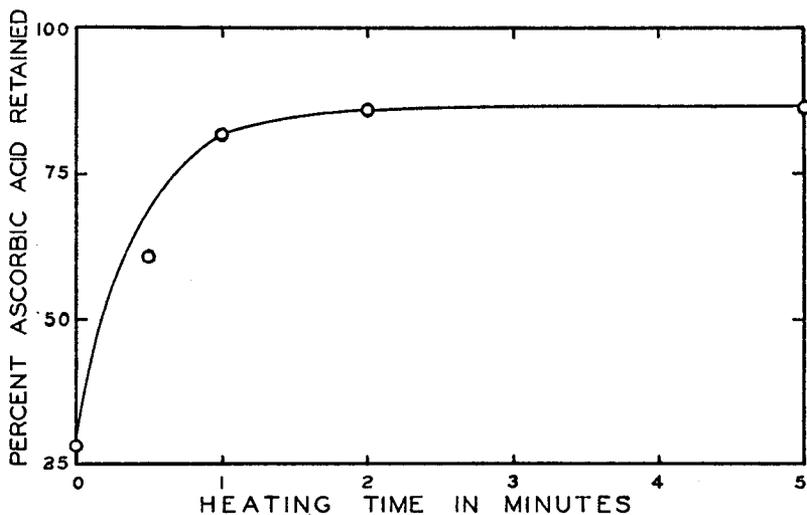


FIG. 3. Retention of ascorbic acid in raw and heated peas frozen at -33° and stored at -7° .

negative. The irreversible reaction product in the fourth and seventh columns of Table II was determined by difference.

The irreversible reaction at pH 3.7 is similar to that occurring in a more nearly neutral solution, and non-antiscorbutic products are formed in both cases (2). This non-antiscorbutic reaction product reduces the indophenol dye in neutral or faintly acid solution. In our experiments the titration was performed in 6 per cent acetic acid, so that the pH of the solution was about 3.0. It is believed that the reaction between the dye and the irreversible form of the first oxidation product of ascorbic acid is not appreciable in strongly acid solution. Hence, this method of analysis for the three forms of ascorbic acid should be valid.

The ascorbic acid disappeared rapidly from the solution in the presence of the enzyme. The decomposition of the ascorbic acid from non-enzymic causes was relatively much slower, only about 17 per cent in 3 hours. There also appears to be a correlation between the proportion of ascorbic acid finally lost for antiscorbutic purposes and enzyme action inasmuch as the loss is much more rapid in the presence of the enzyme.

TABLE II

Decomposition of Ascorbic Acid in Cabbage Extract in Presence and Absence of Ascorbic Acid Oxidase

The results represent the percentage of the initial ascorbic acid content. The initial pH of all the reaction mixtures was 3.7.

Time	With enzyme (not heated)			Without enzyme (heated)		
	Ascorbic acid	Dehydro-ascorbic acid (regenerable)	Decomposed (not regenerable)*	Ascorbic acid	Dehydro-ascorbic acid (regenerable)	Decomposed (not regenerable)*
<i>hrs.</i>						
0	100	0	0	100	0	0
1	3	88	9	91	4	5
3	0	79	21	83	2	15
20	0	40	60	61	15	24

* Physiologically inactive.

DISCUSSION

The fact that many vegetables contain an active ascorbic acid oxidase was to be expected from reference to the literature. It was also expected that this oxidase would be relatively easily inactivated by heating. The enzyme is interesting from the standpoint of the plant biochemist because unquestionably much significance should be attached to the presence and rôle of ascorbic acid and ascorbic acid oxidase in plants. It is considered, however, that ascorbic acid oxidase catalyzes the formation of dehydroascorbic acid, an antiscorbutic agent equal in effectiveness to ascorbic acid; the nutritionist might question the practical significance of the presence of this enzyme in plants.

The authors believe that evidence presented in this paper, as well as several observations previously recorded in the literature, points to the great importance of this enzyme in the preservation

of vegetables. An inspection of Table II shows that at any time the proportion of the dehydroascorbic acid in the mixtures was much larger in the presence than in the absence of the enzyme. By assuming that the rate of decomposition of dehydroascorbic acid is a function of its concentration, one obtains a reasonable explanation of the more rapid loss of physiologically active forms of ascorbic acid in the presence of the enzyme. The rate of decomposition of dehydroascorbic acid, as shown in Table II, is considerably greater than that reported by Wurmser and Sonbeiro (15). We have found that the irreversible decomposition of ascorbic acid in vegetable extracts, even with the enzyme inactivated, is more rapid than in pure aqueous solutions of ascorbic acid of the same concentration and pH.

Further evidence supporting this theory can be found in Fig. 3 in which the ascorbic acid content of raw and blanched peas frozen and stored at -7° is shown. The highest loss of ascorbic acid occurred in the unblanched sample in which, after 2 months of storage, a small proportion of the original ascorbic acid was found in the form of dehydroascorbic acid. The loss of ascorbic acid was less in the sample blanched for half a minute, and in the samples blanched for 1 minute or more no loss was observed except the uniform slow non-enzymic degradation. Again the indirect detrimental effect of the enzyme on the preservation of the ascorbic acid is indicated.

From the large number of cases which could be cited in support of this hypothesis only one will be mentioned here. As early as 1931, Kohman, Eddy, and Gurin (9) reported that minced carrots lost much of their antiscorbutic activity upon standing in air for 1 hour. At the time of these investigations dehydroascorbic acid was unknown. Later work revealed that heating (blanching) practically prevented this loss of ascorbic acid in minced vegetable tissues. The loss of ascorbic acid in a physiologically active form was more rapid where the ascorbic acid had been transformed by enzyme action into dehydroascorbic acid.

SUMMARY

1. Enzymes participating in the oxidation of ascorbic acid have been shown to be generally present in vegetables. Already known to be present in cabbage and squash, it has also been found in

pumpkins, peas, string beans, Lima beans, sweet corn, Swiss chard, carrots, parsnips, and spinach. The relative activity of this enzyme varies greatly in different vegetables.

2. Ascorbic acid oxidase is completely inactivated in vegetables or in their extracts by heating to 100° for 1 minute.

3. The presence of ascorbic acid oxidase is instrumental in the loss of physiologically active forms of ascorbic acid by catalyzing the transformation of this latter into dehydroascorbic acid, which is more readily decomposed by a non-enzymic reaction into a compound having no antiscorbutic activity.

4. Peas frozen after sufficient heat treatment to inactivate the ascorbic acid oxidase retained a much greater portion of their original ascorbic acid content than those having the active enzyme present. Since the ascorbic acid oxidase and catalase appear to be inactivated by heat at the same rate, the commercial practice of establishing the proper blanching time by a test for catalase activity also yields information on the inactivation of the ascorbic acid oxidase.

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