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STUDIES ON THE STABILITY OF L-ASCORBIC ACID (I)

By

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Introduction

L-ascorbic acid (ASA) is added to many diets for the purpose of the enrichment, the protection of oxidation and the prevention of browning. Of course, ASA may be said to be universally distributed, more or less, in vegetables and fruits. The stability differs, however, between ASA in natural diets and that in the synthetic diets, that is, ASA in the former is more stable than that in the latter. ASA in natural diets is first oxidized to dehydroascorbic acid (DHA) by various factors and then DHA produces three reductones (A, B and C) as intermediates in degradation, and among them, reducton A has a very strong antioxidative effect on ASA (1) (2). This is why ASA in natural diets has reasonable stability. ASA in the synthetic diets, on the other hand, is little oxidized to DHA, but decomposed to furfral etc. (3). These reactions are easily influenced by temperature (4) and oxygen (5).

It has been found by several groups of workers (6) (7) that in cooking the kind of diets produces difference in the oxidation-degree of ASA. This may be due to the existence of a protective substance (8) and the amino acids which accelerate or inhibit the oxidation of ASA (9), besides some kinds of metal ions and enzymes in the diets.

As mentioned abve, the oxidation of ASA in the diets is influenced by various factors. Accordingly, it appeared to be of interest to investigate the oxidation aspect of ASA in the diets. The present investigation was undertaken in order to see as a preliminary research the stability of ASA in aqueous solution under the various conditions.

Experimental

Materials

ASA and the other reagents which were used in this experiment were obtained commercially.

Analytical methods

Determination of ASA and DHA; these were determined by indophenol-buthanol method (10).

The activity of ASA oxidase; this was determined by Fujita's method (11).

Procedure

The effect of time; ASA in aqueous solution (20 mg. %) was allowed to stand at 40°, then the remaining ASA and the produced DHA were determined after 0, 20, 40

and 60 minutes, respectively.

The effect of temperature; ASA in aqueous solution (20 mg. %) was allowed to stand at 0°, 20°, 40°, 60° and 80°, respectively, then the remaining ASA and the produced DHA were determined after 60 minutes exactly.

The effect of pH; ASA in aqueous solution (20 mg. %) and the sample solution prepared from a summer orange were allowed to stand in various pH solutions (from 1 to 11) at 40° for 60 minutes exactly, then ASA and DHA were determined.

The effect of oxygen; ASA in aqueous solution (20 mg. %) was assigned to 2 groups as follows; Group I—carried out in an atmosphere of nitrogen at 40° for 60 minutes; Group II— carried out by areration at 40° for 60 minutes. Then the remaining ASA and the produced DHA were determined.

The effect of light illumination; ASA in aqueous solution (20 mg. %) was assigned to 2 groups as follows; Group I—allowed to stand at various pH in a light place exposed to sunshine directly at 40° for 2 hours; Group II—allowed to stand at various pH in a dark place at 40° for 2 hours. Then the remaining ASA and the produced DHA were determined.

The effect of ASA oxidase; the relation between ASA oxidase prepared from Japanese pumpkin and time, temperature, pH or light illumination was observed by estimation of the remaining ASA.

The effect of sodium chloride; sodium chloride (final concentration = from 0.01% to 1.0%) and 0.1 ml. of the prepared ASA oxidase solution were added to 100 ml. of 20 mg. % ASA in buffer solution (pH = 5.5), and the remaining ASA was determined after standing at 40° for 30 minutes by aeration.

Preparation of ASA oxidase solution; the rinds of Japanese pumpkin were ground down into paste with a eartherware-pestle by adding a small quantity of sea-sand in the mortar. Four times volume of distilled water was added to the ground rind of pumpkin, and after mixing up sufficiently the mixture was centrifused for 10 minutes at approximately 3000 r. p. m. Naught point two ml. of about 27% barium acetate solution was added to 10 ml. of the clear solution. After mixing up sufficiently the mixture was filtered, then 0.5 ml. of saturated ammonium sulfate solution was added to 10 ml. of the filtrate. The mixture was centrifused again under the same condition, and then 4.3 gr. of ammonium sulfate was added to 10 ml. of the clear solution. The mixture, moreover, was mixed up sufficiently and centrifused under the same condition. The obtained precipitate was washed with saturated ammonium sulfate solution, then the washed precipitate was dissolved in a little volume of distilled water and centrifused under the same condition. Two ml. of toluene was added to the obtained solution and the mixture was dialyzed for 1 or 2 days with cellophane bag. After dialysis, the mixture was centrifused under the same condition. The obtained clear solution was used as ASA oxidase solution.

Results and Discussion

Since temperature and time have an important effect upon the stability of ASA, it was thought advisable to keep a constant condition for the investigation of autoxidation, photoxidation and enzymic decomposition of ASA. The relations between temperature

or time and the autoxidation of ASA in aqueous solution are shown in Figs. 1 and 2.

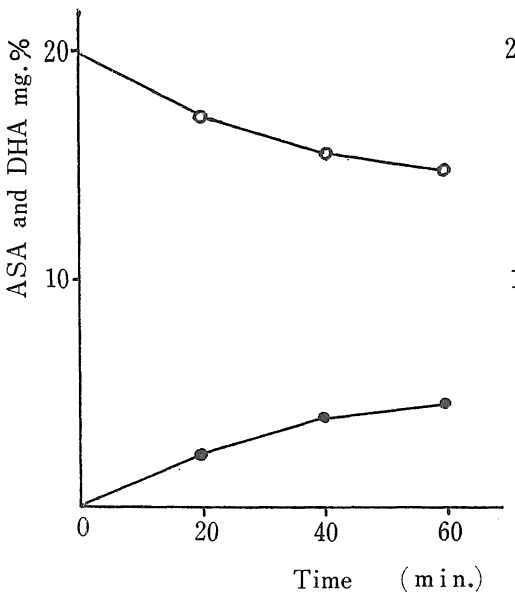

Fig. 1. Effect of time on autoxidation of ASA(at 40°)
○—○ ASA ●—● DHA

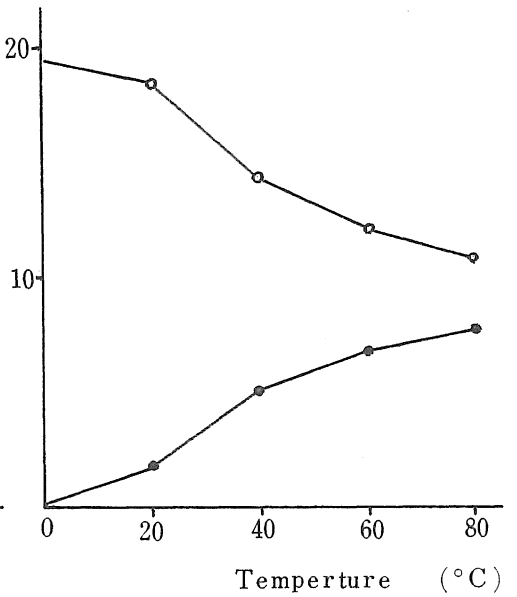

Fig. 2. Effect of temperature on autoxidation of ASA (for 60 min.)
○—○ ASA ●—● DHA

ASA decreased with the passing of time, especially for the first 20 minutes, and the decrease came to be slow after 60 minutes, compared with the degree of decrease after 20 minutes, as shown in Fig. 1. Moreover, the decomposition of ASA was recognized conspicuously at 40° and above, as shown in Fig. 2. In view of the above results, all experiments were carried out under the constant conditions, that is, at 40° for 60° minutes, unless otherwise stated.

On the stability of ASA in various pH solutions

As it seemed reasonable to assume that the autoxidation of ASA to DHA may have relation to pH, the remaining ASA and the producrd DHA in ASA in aqueous solutions which were allowed to stand in various pH solutions were determined. The results are shown in Fig. 3.

As shown in Fig. 3, it was found that ASA was reasonably stable in acidic solutions but unstable in alkalic solutions. Hatano (12) has reported that the decomposition mechanism of ASA is greatly influenced by pH when oxidation or reduction is involved. It was recognized in this experiment that ASA was remarkably stable below pH 3 and decreased rapidly above pH 8. It was expected that the contents of DHA might increase in proportion to the rise of pH as a result of the autoxidation of ASA, but the experimental results disappointed our expectation. Though the contents of DHA increased from pH 4 little by little, the curves of DHA went down slowly from pH 7. It may be concluded from these results that DHA was rapidly degraded in alkalic solutions to 2, 3-diketoglonic acid (DKG) or the other decomposed products, such as, reductones or furfral etc. In order to observe these aspects, ASA and DHA

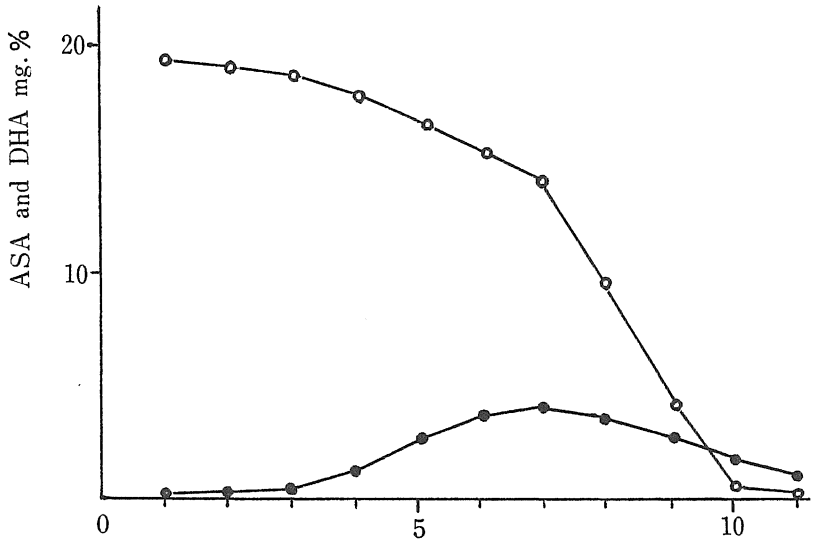

Fig. 3. Effect of pH on autoxidation of ASA
○—○ ASA ●—● DHA

contained in a summer orange allowed to stand at various pH solutions, were determined and the results are summarized in Fig. 4.

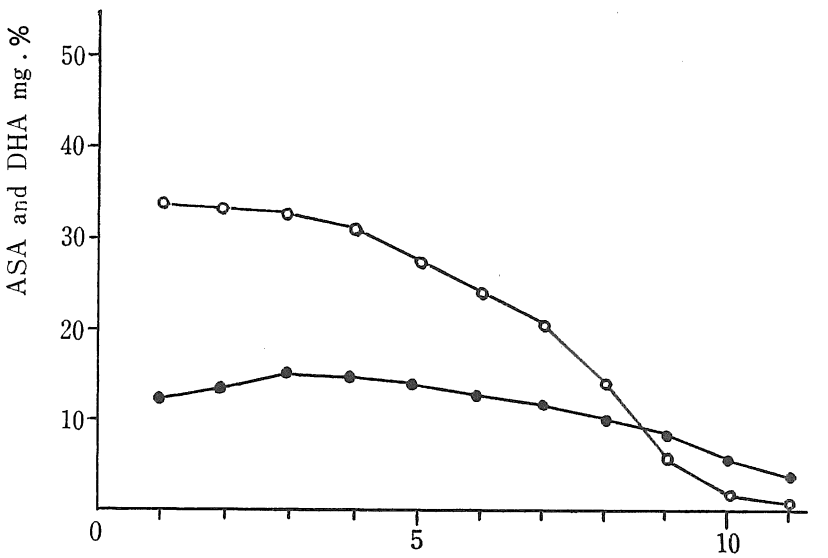

Fig. 4. Effect of pH on the stability of ASA and DHA contained in a summer orange
○—○ ASA ●—● DHA

The results showed that ASA was reasonably stable in acidic solutions and oxidized to DHA rapidly in proportion to the rise of pH. On the other hand, DHA level showed the highest value at pH 3, and decreased slowly in proportion to the rise of pH.

In view of the above facts, the most reasonable conclusion to be drawn from the

available data is that ASA is strongly stable below pH 3 and DHA is most stable at pH 3. It seems most reasonable to conclude, moreover, that DHA drived from ASA is degraded in alkalic solutions to DKG or the other decomposed producfs, such as reductones and furfral etc., because the increase of DHA was not recognized in spite of the decrease of ASA. Terada et al. (13) have reported that DHA was most stable at pH 2.4 and its stability decreased rapidly below pH 2 or above pH 6. The present author has confirmed these findings.

On the stability of ASA for oxygen in various pH solutions

Since oxygen appears to be involved in the oxidation of ASA, it was thought advisable to investigate the effect of oxygen on ASA in various pH solutions.

One group was carried out in an atmosphere of nitrogen, and the other group by aeration for 1 hour. The data are shown in Fig. 5.

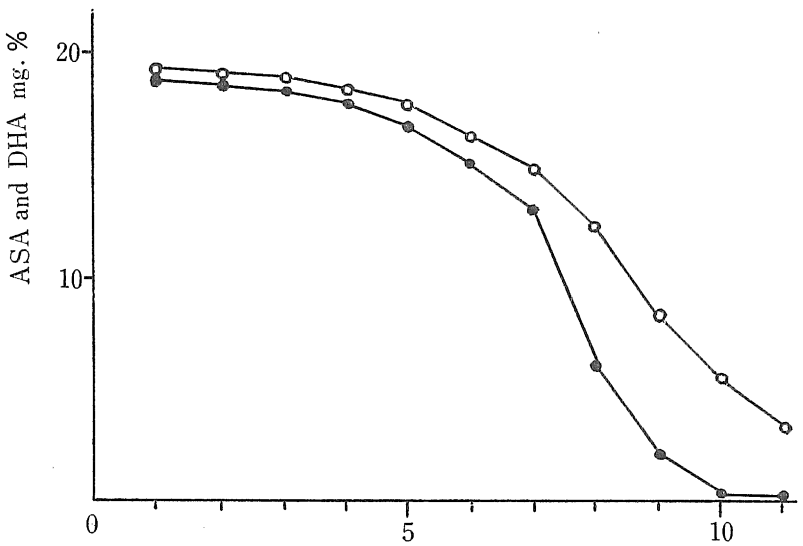

Fig. 5. Effect of oxygen on autoxidation of ASA
○—○ ASA(Nitrogen) ●—● ASA(Aeration)

It was evident from these data that the oxidation of ASA was protected by the presence of nitrogen gas instead of oxygen. This tendency was recognized especially in alkalic solutions. The fact that the presence of nitrogen gas inhibited the oxidation of ASA was in good agreements with the observation of Mori et al. (14). But it seemed that the effect of pH on the stability of ASA was stronger than that of oxygen.

On the stability of ASA by light illumination

It appeared of interest to investigate the photoxidation of ASA, since ASA has the endiol radical having the double bond, and ultraviolet rays was absorbed strongly in the double bond. The effect of radiation of ultraviolet and visible rays on ASA has been studied by several groups of workers (14)~(17) in recent years, but little attention has been paid to the relation between light illumination and pH. In an effort to determine the effect of light illumination, therefore, the examination was made of the photoxidation of ASA in aqueous solution at a light piace exposed to the sunshine for 2 hours in various pH solutions, and it was compared with that allowed to stand at a

dark place for 2 hours. Two solutions of pH 10 and 11 were omitted, because ASA was almost decomposed in their solutions, as shown in Fig. 3. The results are given in Fig. 6.

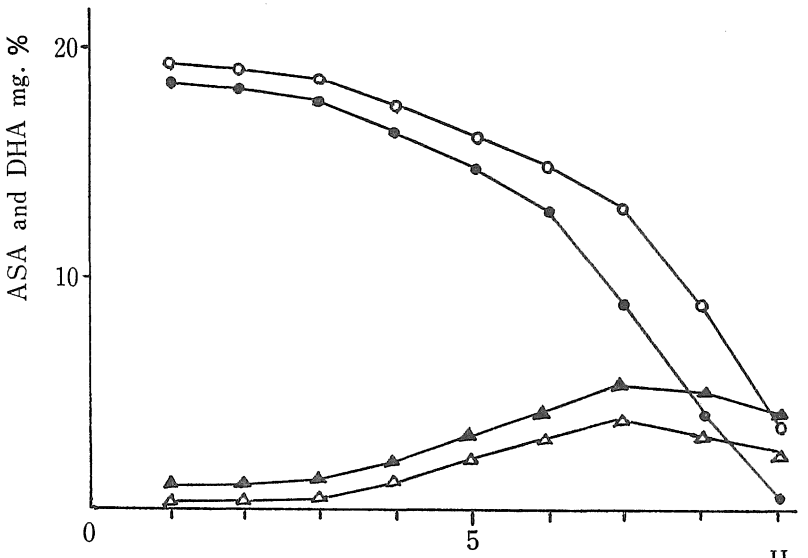

Fig. 6. Effect of light illumination on the stability of ASA
○—○ ASA (Control) ●—● ASA (Illuminated)
△—△ DHA (Control) ▲—▲ DHA (Illuminated)

It was recognized that the effect of light illumination on the stability of ASA in aqueous solution was stronger in alkalic solutions than in acidic solutions. Photoxidation of ASA to DHA was clearly recognized, but the degradation of DHA by light illumination was found little in all the cases. This fact was in good agreement with the observation reported by Kitagawa (15). It was found, therefore, that ASA was oxidized rapidly to DHA by light illumination, especially in alkalic solutions, but DHA was little decomposed in every pH solution.

The effect of ASA oxidase on ASA under the various conditions

In view of the above data, it was found that ASA in aqueous solution was decomposed to some compounds by non-enzymic reactions. It is well known, however, the oxidation of ASA depends upon the contents of ASA oxidase contained in the diets. Interest in this fact, therefore, has led to the suggestion to observe the effect of ASA oxidase for the oxidation of ASA under the various conditions. The results are shown in Figs. 7−10.

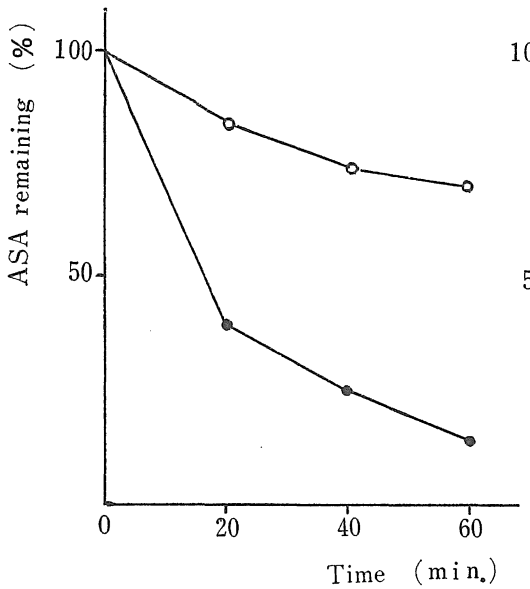

Fig. 7. The relation between ASA oxidase and time
○—○ Control
●—● ASA oxidase was added

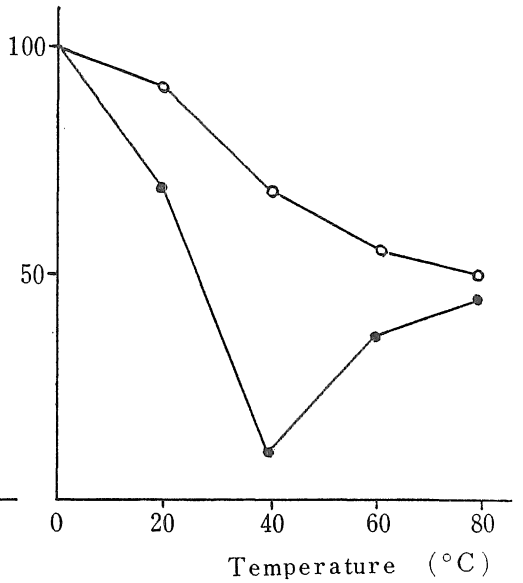

Fig. 8. The relation between ASA oxidase and temperature
○—○ Control
●—● ASA oxidase was added

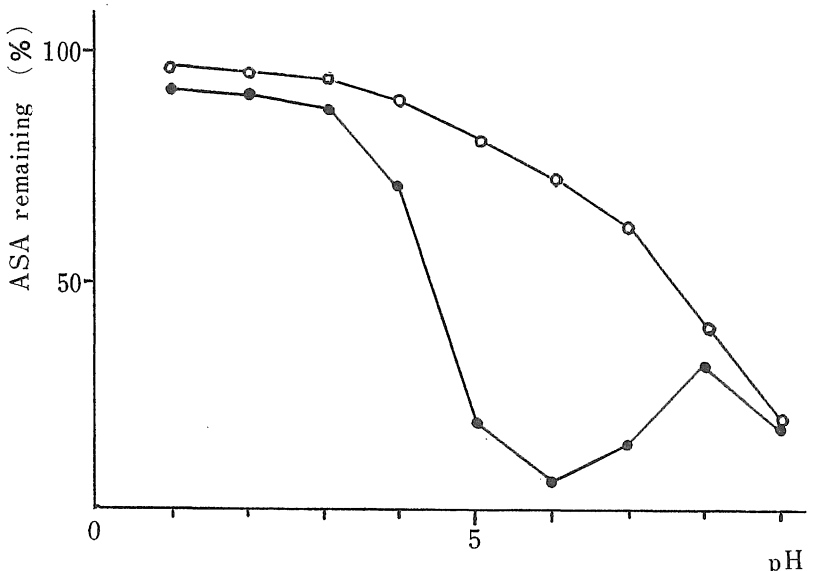

Fig. 9. The relation between ASA oxidase and pH
○—○ Control
●—● ASA oxidase was added

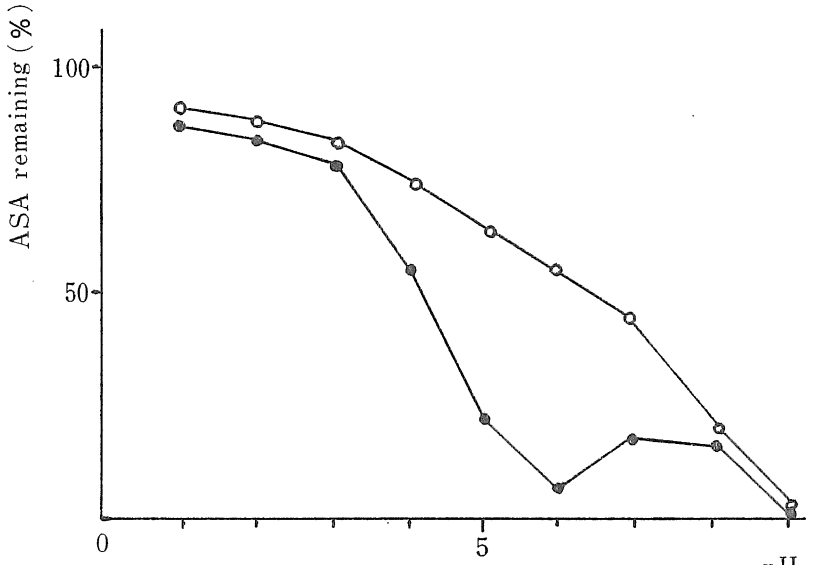

Fig. 10. The relation between ASA oxidase and light illumination
○—○ Control
◍—◍ ASA oxidase was added

As shown in Fig. 7, the level of the remaining ASA with ASA oxidase added descended remarkably compared with that of the control group, and the conspicuous decrease of ASA was recognized at about 40°, as shown in Fig. 8. Accordingly, it seemed that the effect of ASA oxidase on ASA may be the greatest at about 40°. Furthermore, it was found that ASA was remarkably oxidized at pH 5 or 6 by ASA oxidase, as given in Fig. 9. And this tendency was the same with the case of light illumination, as shown in Fig. 10. It may be concluded from these results, therefore, that the photoxidation of ASA is independent of the presence of ASA oxidase.

In an effort to determine the effect of ASA oxidase, the enzymic activity was observed under the various conditions of temperature and pH, with the consideration that ASA oxidase was closely connected with them. The results are summarized in Figs. 11 and 12.

Consequently, it was found that the activity of ASA oxidase was remarkably recognized between 35° and 45°, and the maximum value was obtained at 40°, as given in Fig. 11. The enzymic activity decreased greatly below 30° or above 50°. The optimum pH of ASA oxidase was 5.5 and the activity was little recognized below 3 or above 8, as shown in Fig. 12. On the basis of these data, it seemed reasonable to assume that ASA oxidase showed the greatest activity at 40° and pH 5.5.

It has been reported by several groups of workers (18)~(20) that some inorganic- or organic-compounds inhibit the activity of ASA oxidase. ASA oxidase and polyphenol oxidase are in close connection with the browning of diets. It is well known, on the other hand, that sodium chloride is added to the fresh fruits in order to inhibit the tyrosinase which occurs the browning of them. And sodium chloride is a general seasoning in cooking of diets. Accordingly, it appeared to be of interest to investigate

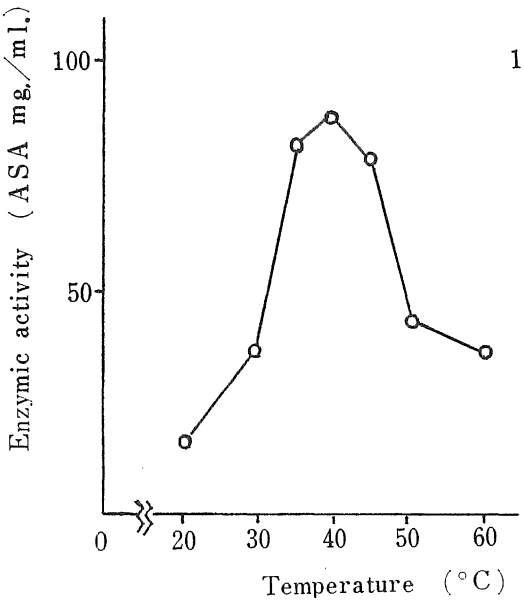

Fig. 11. Effect of temperature on ASA oxidase

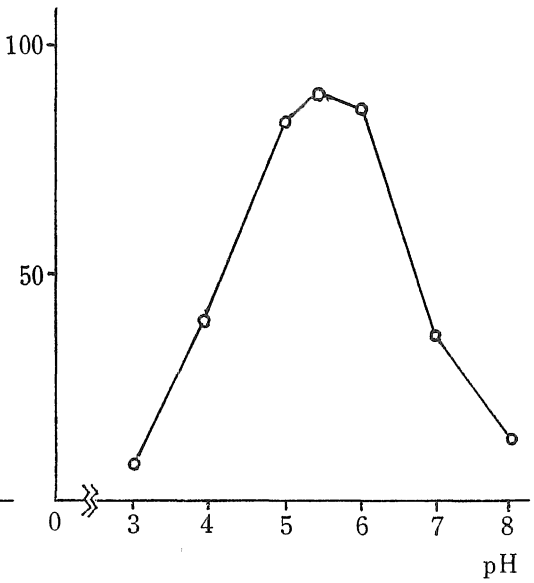

Fig. 12. Effect of pH on ASA oxidase

further the scope of the effect of sodium chloride on ASA oxidase. The results are summarized in Table I.

Table I. The effect of sodium chloride on ASA oxidase

Substances	ASA remaining (%)
Control (ASA 20 mg. in 100 ml. of pH 5.5 buffer solution)	74.3
Control + NaCl(1.0%*)	73.6
Control + ASA oxidase 0.1 ml.	7.4
Control + ASA oxidase + NaCl (0.01%*)	19.3
Control + ASA oxidase + NaCl (0.1 %*)	28.2
Control + ASA oxidase + NaCl (0.5 %*)	34.3
Control + ASA oxidase + NaCl (1.0 %*)	38.7

40°, 30 min., aeration * = Final concentration

As shown in Table I, it was found that sodium chloride inhibited clearly the oxidation of ASA in proportion to the rise of its concentration in the presence of ASA oxidase, though the addition of sodium chloride to ASA in buffer solution had no effect on the oxidation of ASA at all. In this experiment, the oxidation of ASA by ASA oxidase was strongly recognized because of long time, but was considerably protected in the final concentration of 0.5% sodium chloride. This concentration does not interfere with the daily cooking of diets. It seems, therefore, that addition of sodium chloride to diets is useful for the protection of the oxidation of ASA or the

prevention of browning.

Summary

The present investigation was undertaken to see as a preliminary research the stability of ASA in aqueous solution under the various conditions. The following results were obtained.

It was found that ASA was remarkably stable below pH 3 and decreased rapidly above pH 8, especially was almost decomposed at pH 10 or 11. It seemed most reasonable to conclude, on the other hand, that DHA is most stable at about pH 3.

Though the presence of oxygen accelerated the autoxidation of ASA, it seemed that the effect of pH on the stability of ASA was stronger than that of oxygen.

Photoxidation of ASA to DHA by light illumination was recognized stronger in alkalic solutions than in acidic solutions. But DHA was little decomposed in every pH solution.

It was found that the activity of ASA oxidase was remarkably recognized between 35° and 45° and the maximum value was obtained at 40°. The optimum pH of ASA oxidase was 5.5 and the activity was little recognized below pH 3 or above pH 8. The photoxidation of ASA, moreover, may be independent of the presence of ASA oxidase.

The oxidation of ASA oxidase was considerably protected in the final concentration of 0.5% sodium chloride. This concentration does not interfere with the daily cooking of diets. It seems, therefore, that addition of sodium chloride to diets is useful for the protection of the oxidation of ASA.

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