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Studies on the Natural Inhibitors Contained in Citrus Fruits Against Ascorbic Acid Oxidase

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Introduction

It was already reported that the natural inhibitors against ascorbic acid oxidase are contained in tomato, strawberry, egg-apple, lettuce, etc. by Inagaki, Fukuba and others[1]-[3]. That the inhibitors are not compound of one kind but of several kinds are already recognized, namely volatile organic compound for tomato, inorganic anion, especially chromate ion, for strawberry and anthocyanin pigment (nasunin) and globulin for egg-plant.

Ascorbic acid contained in oranges is well known to be very stable for oxidation, comparing with simple ascorbic acid solution[4].

This would be due to the natural inhibitor against ascorbic acid oxidase and also for autoxidation of this vitamin contained in these.

It is considered that some materials contained in oranges, such those of saccharids, acids, bitter materials, hesperidin, naringin, pectin, vitamin B_1, B_6, and carotene might have inhibitory activity against ascorbic acid oxidase[4].

The authors[5] reported the presence of some inhibitor contained in oranges and that this compound was readily oxidized during the preparation of orange juice by mixer, while the nature of this compound itself was not yet revealed.

Preliminary Experiments

Introduction

It has reported that gelatin solution works as a stabilizer for the ascorbic acid oxidase, so it was tried that the influence of the concentration of gelatin solution for the stability of the ascorbic acid oxidase.

The effect of gelatin solution for the stability of ascorbic acid oxidase.

Experimentals—

(1) Procedures

All determinations were carried out with Warburg's manometer at 30°C.

Oxidation of ascorbic acid appears on manometer as oxygen absorption.

Inhibitory activity can be known by the difference of the volume of oxygen absorbed between the reaction media added inhibitor and the one without inhibitor.

The flask with one side arm was used.

Ascorbic acid, buffer, and gelatin solutions were pipetted into the main room, and enzyme solution into the side arm. The composition of the reaction media is cited in later tables. And the total was made up 2.5 ml or 3 ml.

The flasks were incubated for 10 minutes in the constant water bath kept at 30°C for the sake of temperature equilibrium, then, the contents of the side arms were poured into the main rooms. The absorption of oxygen in 30 minutes was measured at intervals of 5 minutes.

(2) Reagents

A) *l*-Ascorbic acid solution

0.5866 g of crystalline *l*-ascorbic acid are dissolved with distilled water and then the volume is made up to 100 ml with the same distilled water. The concentration of this solution with *l*-ascorbic acid is N/30.

B) Buffer solution

MacIlvaine's phosphate-citrate buffer (pH 5.7) is used.

C) Ascorbic acid oxidase solution.

Cucumber is grated with china grater and squeezed by hand. The juice thus obtained is used as enzyme solution.

D) Gelatin solution

Jellice (commercial name of gelatin) is dissolved in hot water to make 0.3%, 0.5%, 1% solution, respectively.

Results

Table 1. Influence of gelatin solution for the stability of ascorbic acid oxidase.

Flask No. / Composition of the reaction media	I	II	III	IV
Ascorbic acid soln.	0.5 (ml)	0.5 (ml)	0.5 (ml)	0.5 (ml)
Buffer	2.0	1.0	1.0	1.0
Gelatin soln. 0.3%		0.5		
0.5%			0.5	
1%				0.5
Enzyme soln.	0.5	0.5	0.5	0.5
5 (mins.)	76.4 (μl)	74.2 (μl)	67.9 (μl)	60.5 (μl)
10	135.8	145.6	149.9	132.0
15	140.7	148.4	152.7	137.5
20	141.5	151.1	152.7	140.2
25	144.3	151.1	155.5	143.0
30	144.3	151.1	155.5	143.0

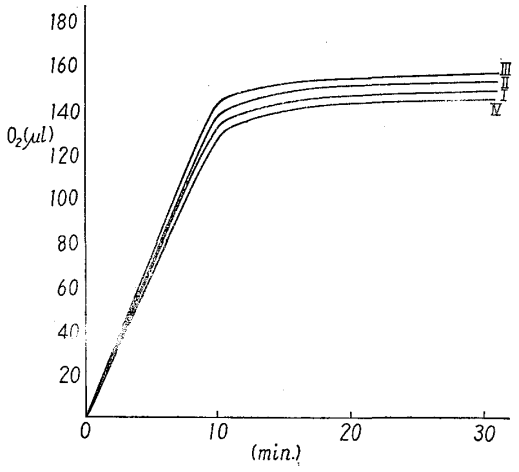


Fig. 1.

Discussion

The result, shown in Fig. 1, indicates the amounts of oxygen absorbed was a little greater with the reaction media added gelatin solution than with the reaction media without gelatin solution, but the clear difference could not be observed. For the gelatin concentration, 0.5% solution showed the greatest oxgen absorption. 1% solution showed less oxygen absorption than the solution without gelatin.

From this result, it can be concluded that the addition of the 0.3–0.5% gelatin solution to ascorbic acid oxidase has a little effect to stabilize ascarbic acid oxidase, therefore, 0.5% gelatin solution was added in the reaction media in the experiments.

Main Experiments

1. The natural inhibitor contained in summer oranges against pumpkin ascorbic acid oxidase

Introduction

There are many kinds of oranges, but some kinds of are not always obtained. It is restricted with season. At first, summer oranges were selected which were easily got from spring to summer, and the presence of the natural inhibitor in it was pursued.

Experimentals—

(1) Procedures

Procedures were almost the same as cited in the preliminary part.

In this part, in addition to the reaction media of the preliminary one, the orange juice squeezed by hand or crushed with the electric mixer, the dialysate of juice against water, the non-dialysate of juice, the concentrate of the dialysate, the distillate of the concentrate of the dialysate, the solutions passed through anion-exchange resin column, and cation-exchange resin column, the ash solution of the orange juice, the ash solutions passed through anionexchange resin column and cation-exchange resin, column, etc. were added in the main room, respectively.

(2) Reagents

A) Ascorbic acid solution

The same one used in the preceding experiment.

B) Buffer solution

The same one used in preliminary experiment. But jellice was added in it to make 0.5% gelatin solution.

C) Ascorbic acid oxidase solution

The oxidase extracted from the pericarp of pumpkin in used. The procedures of extraction are cited in Table A.

Outline of the studies

The outline of the studies is as following. At first, it was investigated the difference of inhibitory activity of the juice squeezed by

Outline of the studies

(+), existence of inhibitory activity

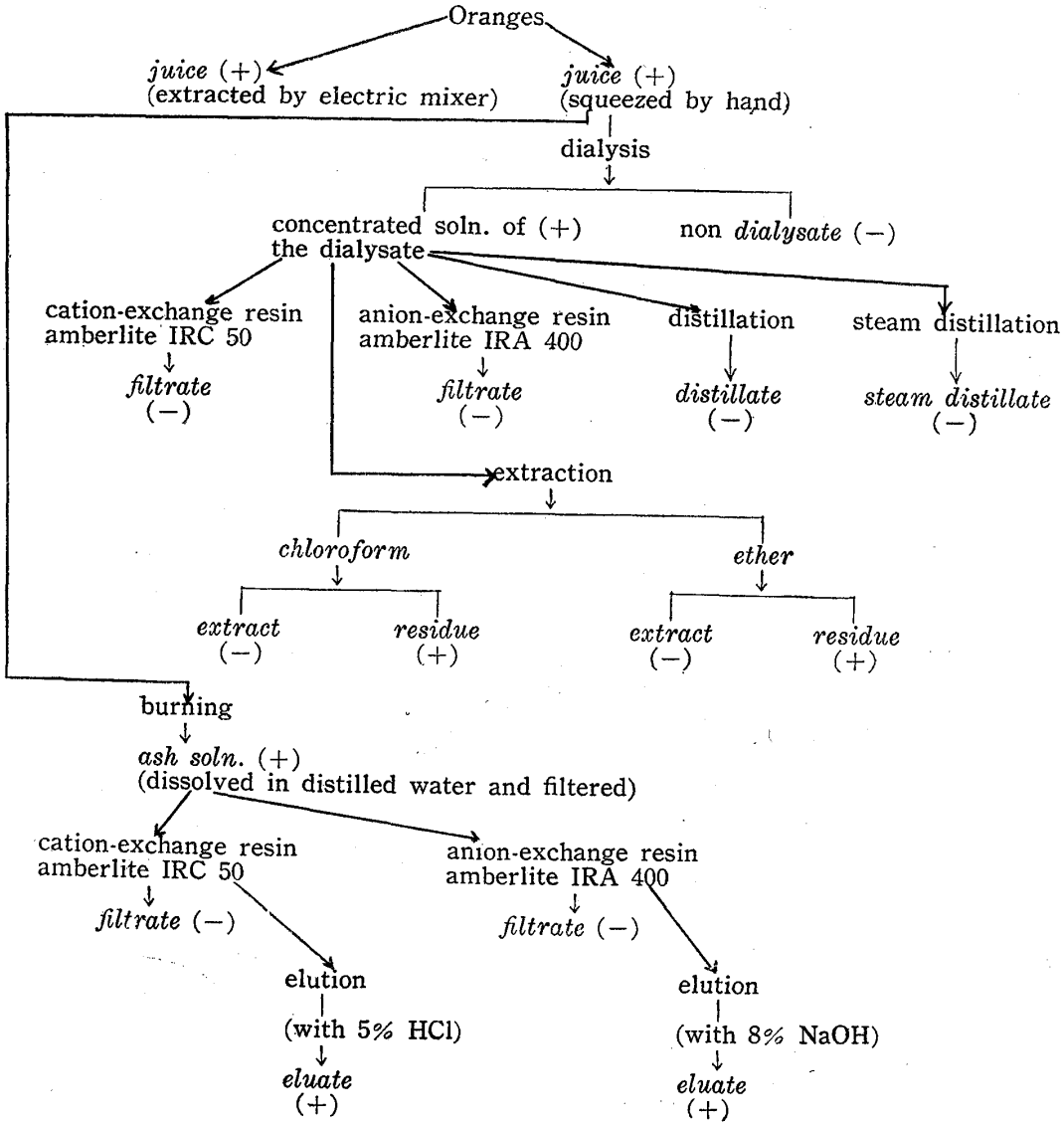
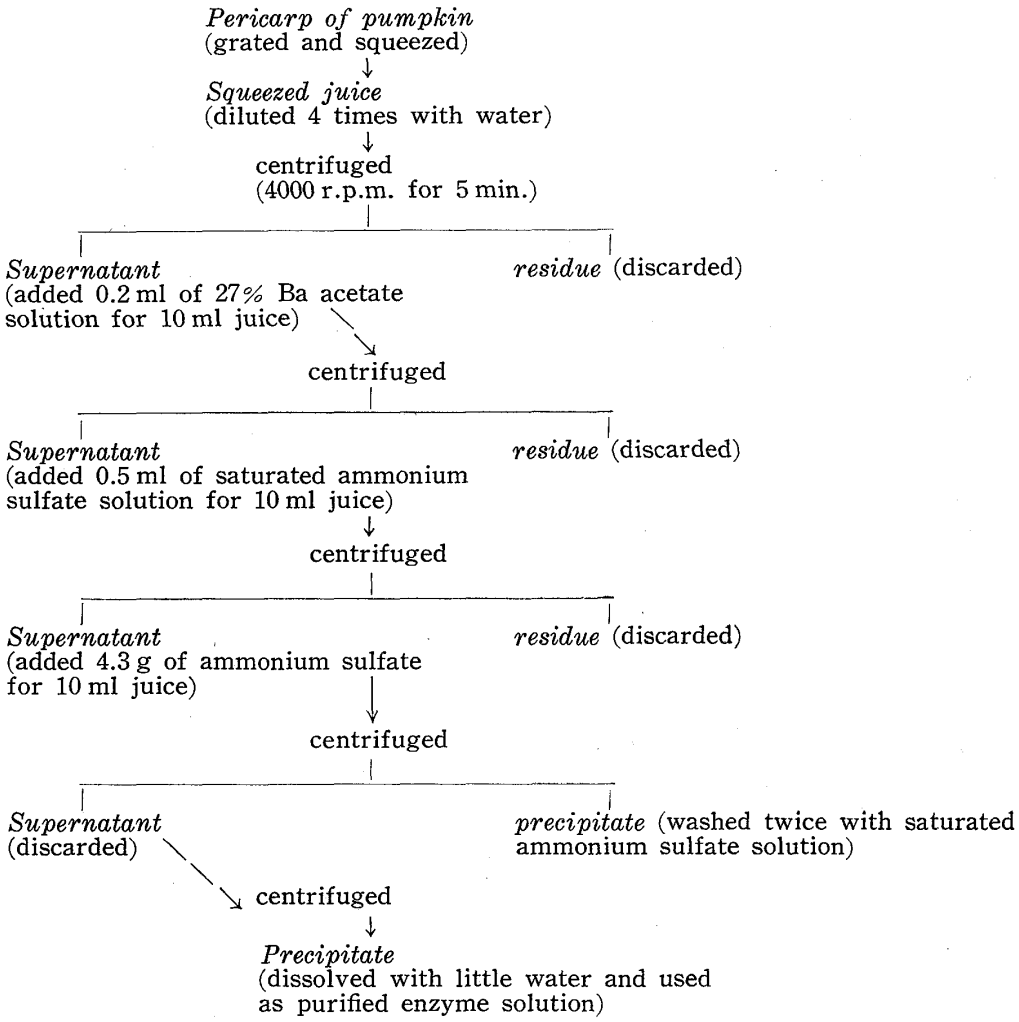


Table A. Extraction and purification of ascorbic acid oxidase from pumpkin.



hand and the juice crushed with electric mixer. Then, the juice squeezed by hand was dialyzed and separated to the dialysable and the non-dialysable fraction.

As the inhibitory activity remained in the concentrate of dialysate, this solution was distilled. But the inhibitory activity was not remained in the distillate. Then, the concentrate of dialysate was passed through anion and cation-exchange resin columns.

The inhibitory activity was not recognized in the filtrate. Next, it was investigated the extraction of the concentrate of the dialysate with ether and chlroform, but the inhibitor was not extracted with those.

Then, the juice was concentrated and burnt into ashes, the inhibitory activity was recognized in ash solution.

Next, ash solution was passed through cation and anion-exchange resin columns.

The inhibitor adsorbed on these resin columns was eluted with 5%

HCl for cation-exchange resin and 8% NaOH for the anion-exchange resin column.

(Experiment I)

It was already reported by C. Inagaki, *et al*[5] that the natural inhibitor is the material readily oxidized in contact with air. So, the experiment on this point was further done, at first, using the juice squeezed by hand and the one crushed with electric mixer.

And it was examined whether the difference of the inhibitory activity appears between the former and the latter.

Results

Table 2. Comparison of the inhibitory activities of the juice squeezed by hand with that crushed with electric mixer.

Flask No.			
Composition of the reaction media	I	II	III
Ascorbic acid soln.	0.5(ml)	0.5(ml)	0.5(ml)
Buffer soln.	1.5	1.0	1.0
*Juice by hand		0.5	
by mixer			0.5
Enzyme soln.	0.5	0.5	0.5

* The juice squeezed by hand: the sarcocarp of oranges was put in the gauze sack and squeezed by hand. The juice crushed with the electric mixer: the sarcocarp of oranges was put in the electric mixer and mixed for 3 minutes.

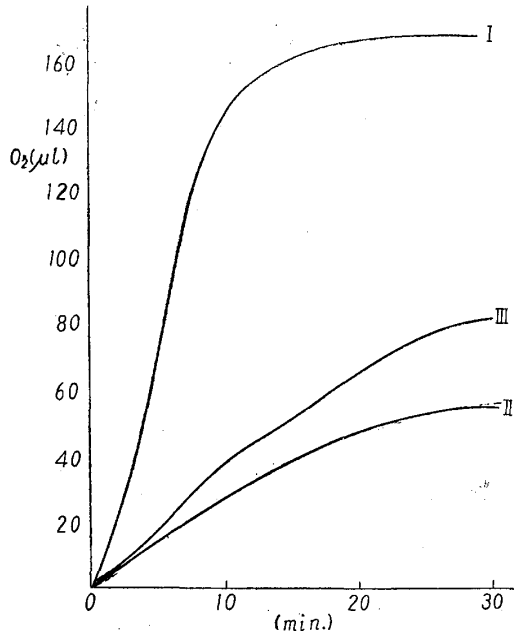


Fig. 2.

Discussion

It is clear, from the Fig. 2, that the inhibitory activity of the juice squeezed by hand is more powerful than that of the juice mixed with electric mixer. This result, the inhibitory activity of the juice by mixer was degraded, indicates, as already reported, the inhibitor is readily oxidized.

Although the inhibitory activity of the juice by mixing for 3 minutes by machine is degraded as compared with the juice squeezed by hand, pretty powerful inhibitory activity still remains in it.

It is necessary to investigate whether the inhibitory activity of the juice would be more destructed during the mixing for longer period or not.

(Experiment II)

On the previous experiment, it was certified that the inhibitor is the material of readily oxidized. Then, it was tried that the various time of mixing, namely, 3, 5, and 10 minutes, and was observed if the difference of the inhibitory activity of these juice appears.

Results

Table 3. Influence of mixing period on the inhibitory activity of orange juice.

Composition of the reaction media	Flask No. I	II	III	IV	VI
Ascorbic acid soln.	0.5 (ml)	0.5 (ml)	0.5 (ml)	0.5 (ml)	0.5 (ml)
Buffer soln. by hand	2.0	1.0	1.0	1.0	1.0
Juice by hand		1.0			
by mixer 3 mins.			1.0		
〃 5 〃				1.0	
〃 10 〃					1.0
Enzyme soln.	0.5	0.5	0.5	0.5	0.5

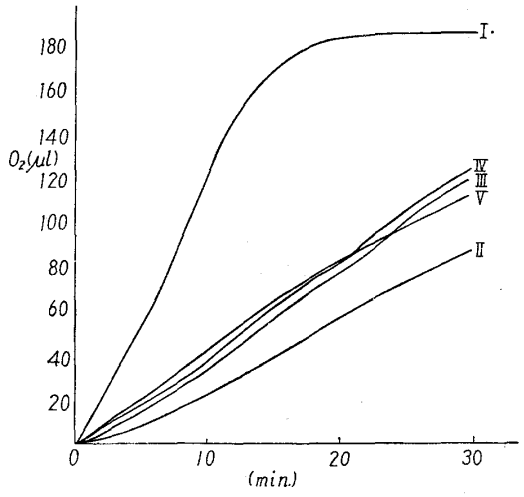


Fig. 3.

Discussion

The result, shown in Fig. 3, indicates that the difference of the inhibitory activity occurred by the variation of the mixing time was scarcely observed. The result showed, too, the one of the inhibitors which was readily oxidized, was completely oxidized within 3 minutes' mixing.

That completely oxidized juice remained considerable inhibitory activity yet, suggests the presence of some other materials as inhibitors which are stable for oxidation.

(Experiment III)

Next, the following experiment was tried in order to examine the presence of the inhibitor which is stable for oxidation.

The juice squeezed by hand was poured into the celophane sack and dialyzed against distilled water during the process for 24 hrs. which was exchanged sometimes. Then, the inhibitory activity of the non-dialysate was examined.

Results

Table 4. Comparison of the inhibitory activity of the juice squeezed by hand with the non-dialysate of juice.

Flask No. / Composition of the reaction media	I	II	III
Ascorbic acid soln.	0.5(ml)	0.5(ml)	0.5(ml)
Buffer soln.	2.0	1.0	1.0
Juice			1.0
Non-dialysate		1.0	
Enzyme soln.	0.5	0.5	0.5

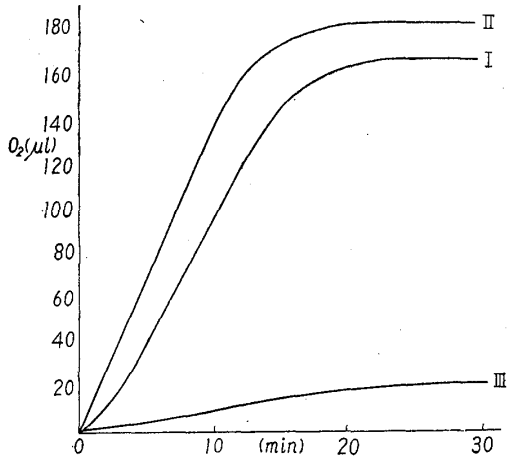


Fig. 4.

Discussion

The non-dialysate of orange juice, have lost its inhibitory effect entirely for the oxidase, from this result, it is assumed that the stable natural inhibitor against ascorbic acid oxidase would be compounds of low molecular.

To clarify on this point, it is necessary to examine whether the dialysate has the inhibitive activity or not.

(Experiment IV)

The dialysate which was obtained by the same procedures as the previous ones was concentrated *in vacuo*.

The inhibitory activity of the concentrate was examined.

Results

Table 5. Comparison of the inhibitory activity of the concentrate obtained from the dialysate of orange juice.

Flask No. / Composition of the reaction media	I	II	III
Ascorbic acid soln.	0.5(ml)	0.5(ml)	0.5(ml)
Buffer soln.	2.0	1.0	1.0
Juice		1.0	
Concentrate			1.0
Enzyme soln.	0.5	0.5	0.5

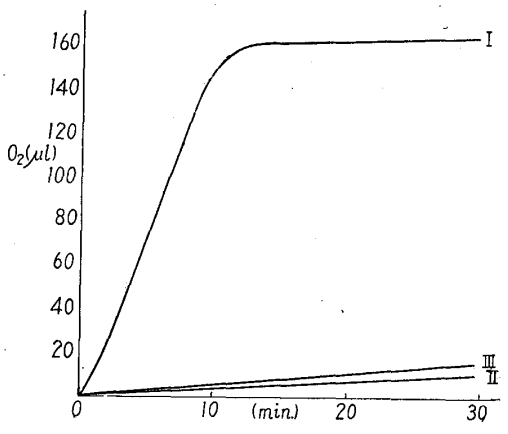


Fig. 5.

Discussion

Inhibitory activity was found to be recovered in the concentrate of the dialysate of juice.

From this result, it is assumed that the inhibitor would be a compound of low molecular.

(Experiment V)

The inhibitor contained in the concentrate of the dialysate of juice was further investigated. The concentrate was distilled with steam. Then, the inhibitory activity of the steam distillate was examined.

Results

Table 6. Comparison of the inhibitory activity of the steam distillate.

Flask No. / Composition of the reaction media	I	II	III
Ascorbic acid soln.	0.5(ml)	0.5(ml)	0.5(ml)
Buffer soln.	2.0	1.0	1.0
Juice			1.0
Distillate		1.0	
Enzyme soln.	0.5	0.5	0.5

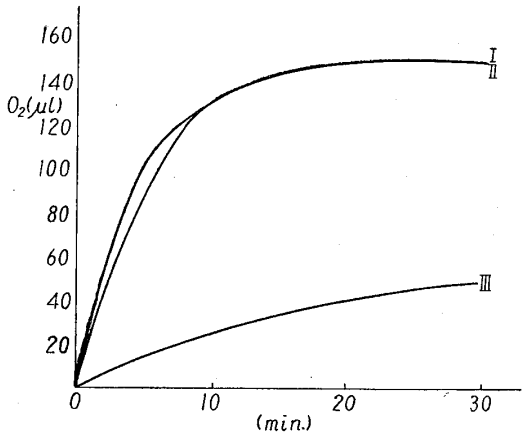


Fig. 6.

Discussion

The result, shown in Fig. 6, indicates that the steam distillate of the concentrate of the dialysate remains no inhibitory activity.

(Experiment VI)

The same concentrate used in experiment V was distilled directly. The inhibitory activity of the distillate was examined.

Results

Table 7. Comparison of the inhibitory activity of the distillate.

Flask No. / Composition of the reaction media	I	II	III
Ascorbic acid soln.	0.5(ml)	0.5(ml)	0.5(ml)
Buffer soln.	2.0	1.0	1.0
Juice		1.0	
Distillate			1.0
Enzyme soln.	0.5	0.5	0.5

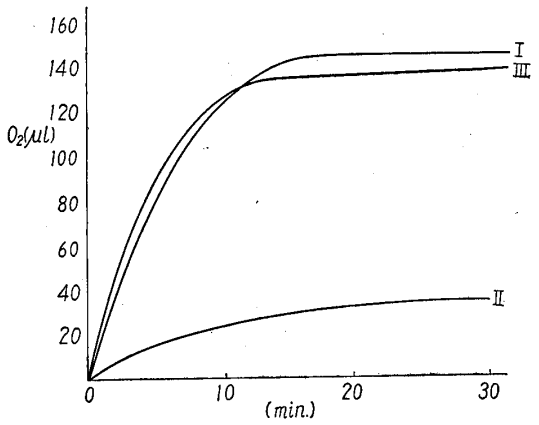


Fig. 7.

Discussion

The result, shown in Fig. 7, showed the direct distillate of the concentrate lost this activity.

From these results of experiment V and VI, it was possible to conclude that the inhibitor would not be the volatile substances.

(Experiment VII)

It has been reported[4] that terpene contained in the essential oil in the pericarp of summer orange may concern the mechanism of this inhibition. So, this time, the pericarp of the summer orange was cut into small pieces, and distillated with steam.

Then, the inhibitory activity of the steam distillate was examined.

Results

Table 8. Comparison of the inhibitory activity of the distillate of pericarps of summer oranges.

Flask No. / Composition of the reaction media	I	II	III
Ascorbic acid soln.	0.5(ml)	0.5(ml)	0.5(ml)
Buffer soln.	2.0	1.0	1.0
Juice		1.0	
Distillate			1.0
Enzyme soln.	0.5	0.5	0.5

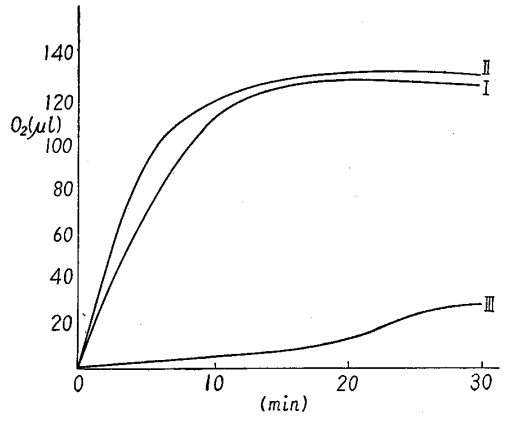


Fig. 8.

Discussion

From this result, it can be determined that terpene has no inhibitory activity. From the results of experiments V–VII, it is clarified that inhibitor is not valatile substances.

(Experiment VIII)

The concentrate of the dialysate was passed through the column of anion-exchange resin, amberlite IRA 400, at the rate of 2 cc/min.

And the inhibitory activity of the solution passed through was examined.

Results

Table 9. Comparison of the inhibitory activity of the solution passed through anion-exchange resin column.

Flask No. / Composition of the reaction media	I	II	III
Ascorbic acid soln.	0.5(ml)	0.5(ml)	0.5(ml)
Buffer soln.	2.0	1.0	1.0
Juice		1.0	
Soln. passed through anion exchange resin column			1.0
Enzyme	0.5	0.5	0.5

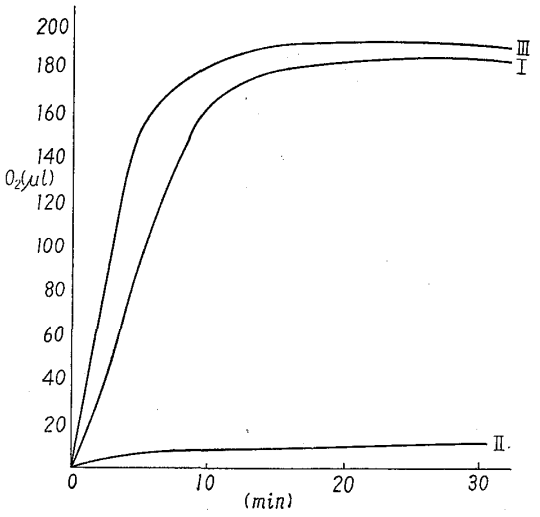


Fig. 9.

Discussion

As distinct from Fig. 9, the concentrate of the dialysate has powerful inhibitory activity but after passing through anion-exchange resin column, it has lost inhibitory activity entirely. From this result, it can be concluded that inhibitor is the substance adsorbed on anion-exchange resin.

The substance adsorbed on anion-exchange resin are organic or inorganic anions, therefore it must be necessary to determine the nature of the inhibitor whether it is a inorganic or a organic anion.

(Experiment IX)

In order to examine whether the inhibitor is organic substance or inorganic one, summer orange juice was concentrated *in vacuo* to jelly, then, put in a evaporating dish and carbonized, next, burnt to ashes in

the muffle, 0.3 g of ash was dissolved with 10 ml of distilled water, then, filtered with filter paper. And the filterate was examined of its inhibitory activity.

Results

Table 10. Inhibition of the ascorbic acid oxidase's action by the addition of the ash solution from summer oranges.

Flask No. / Composition of the reaction media	I	II
Ascorbic acid soln.	0.5(ml)	0.5(ml)
Buffer soln.	2.0	1.0
Ash soln.		1.0
Enzyme soln.	0.5	0.5

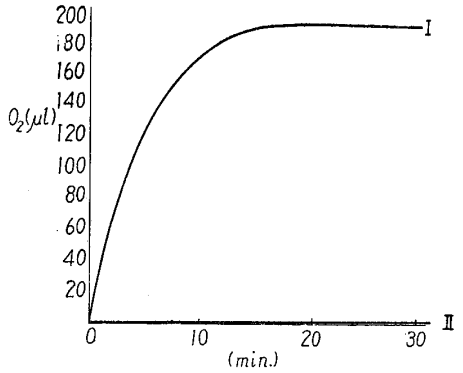


Fig. 10.

Discussion

From the result, ash solution made from summer orange juice was found to remain powerful inhibitory activity still.

Ascorbic acid oxidase's action was inhibited completely and the oxygen absorption was not observed entirely by the addition of the ash solution. Considering the previous results and this result, it became clear that the inhibitor is due to a inorganic anion adsorbed on anion-exchange resin.

Following these experiments, it is desirable to pursue the inhibitor contained in summer oranges, but summer oranges could not be obtained for the sake of out of season, so the experiment using summer orange was stopped. Next, the experiment using mandarin oranges was tried.

2. The natural inhibitor contained in mandarin oranges against as corbic acid oxidase

Introduction

In part 2, the same experiment as previous part, using mandarin oranges, instead of summer oranges, was tried and pursued the nature of the inhibitor, further.

Experimentals—

Procedures and Reagents were all fixed on the same ones as those of part 1.

(Experiment I)

The difference of the inhibitory activity of the juice obtained by hand and the one by mixer, was investigated with the conditions same as those of part 1. The juice obtained by mixer was mixed for 3 minutes.

Results

Table 11. Comparison of the inhibitory activity of the juice squeezed by hand from mandarin oranges with the one crushed with mixer.

Flask No. / Composition of the reaction media	I	II	III
Ascorbic acid soln.	0.5(ml)	0.5(ml)	0.5(ml)
Buffer soln.	2.0	1.0	1.0
Juice by hand		1.0	
by mixer			1.0
Enzyme soln.	0.5	0.5	0.5

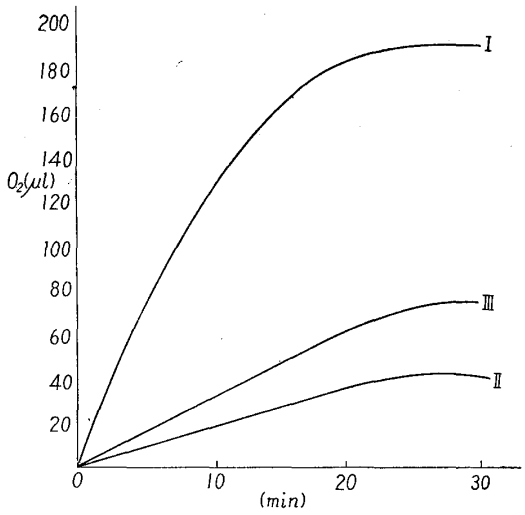


Fig. 11.

Discussion

As same in the experiment using summer oranges the inhibitor in the juice crushed with mixer was degraded to some extent, as compared with the juice squeezed by hand.

When the period of mixing was lengthen from 3 minutes to 5 minutes, the both inhibitory activity were about the same.

And it would be supposed that the juice would be mixed more long time, the same inhibitory curves like as the case of summer orange juice would be obtained.

(Experiment II)

The mandarin orange juice squeezed by hand was dialyzed against distilled water for 24 hrs. with the celophane sack.

And the inhibitory activity of the non-dialysate was examined.

Results

Table 12. Comparison of the inhibitory activity of the non-dialysable fraction of juice.

Composition of the reaction media	Flask No. I	II	III
Ascorbic acid soln.	0.5(ml)	0.5(ml)	0.5(ml)
Buffer soln.	2.0	1.0	1.0
Juice		1.0	
Non-dialysable fraction of juice			1.0
Enzyme soln.	0.5	0.5	0.5

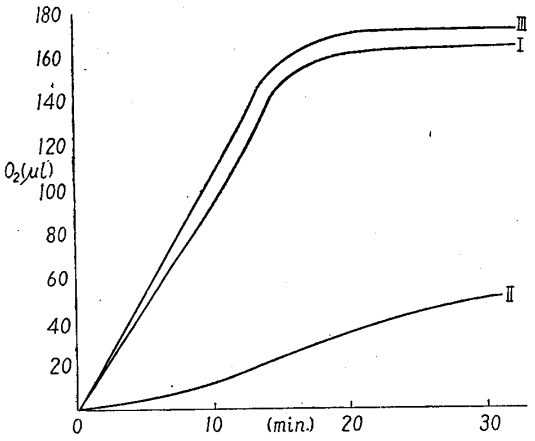


Fig. 12.

Discussion

The result, shown in Fig. 12, suggested that non-dialysate of the mandarin orange juice remained inhibitory activity. This result, was the same one observed in the experiment with summer oranges.

(Experiment III)

As previous experiment, the juice squeezed by hand was dialyzed against distilled water for 24 hrs. with the celophane sack.

Then the dialysate was concentrated to about 1/2 or 1/3 volume of the original one. The inhibitory activity of the concentrate was examined.

Results

Table 13. Comparison of the inhibitory activity of the concentrate of the dialysate.

Composition of the reaction media	Flask No. I	II	III
Ascorbic acid soln.	0.5(ml)	0.5(ml)	0.5(ml)
Buffer soln.	2.0	1.0	1.0
Juice		1.0	
Concentrate of the dialysate			1.0
Enzyme soln.	0.5	0.5	0.5

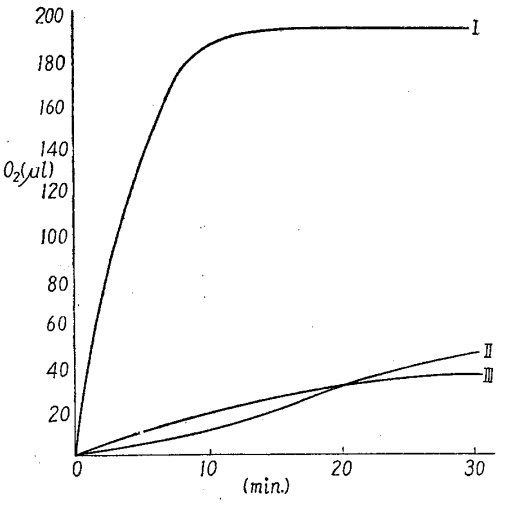


Fig. 13.

Discussion

Inhibitory activity was found to be recovered in the concentrate of the dialysate of the mandarin orange juice. From this result, it is concluded that inhibitor is a compound of low molecular, as same as the inhibitor contained in summer oranges.

(*Experiment IV*)

To examine the presence of the inhibitor contained in the concentrate of the dialysate, this was distilled with steam, and the inhibitory activity of the steam distillate was examined.

Results

Table 14. Comparison of the inhibitory activity of the steam distillate.

Flask No. / Composition of the reaction media	I	II	III
Ascorbic acid soln.	0.5(ml)	0.5(ml)	0.5(ml)
Buffer soln.	2.0	1.0	1.0
Juice		1.0	
Distillate			1.0
Enzyme soln.	0.5	0.5	0.5

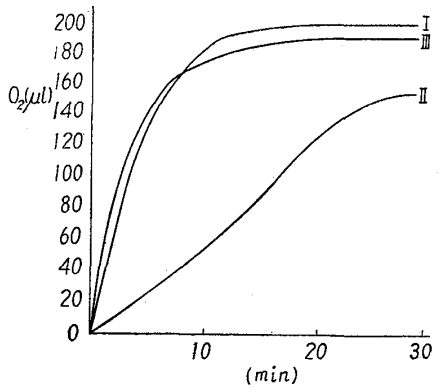


Fig. 14.

Discussion

It was clear, from the result, that the steam distillate of the concentrate of the dialysate lost its inhibitory activity.

(*Experiment V*)

The concentrate of the dialysate was distilled directly. Then the inhibitory activity of the distillate was examined.

Results

Table 15. Comparison of the inhibitory activity of the distillate.

Flask No. / Composition of the reaction media	I	II	III
Ascorbic acid soln.	0.5(ml)	0.5(ml)	0.5(ml)
Buffer soln.	2.0	1.0	1.0
Distillate		1.0	
Concentrate of the dialysate			1.0
Enzyme soln.	0.5	0.5	0.5

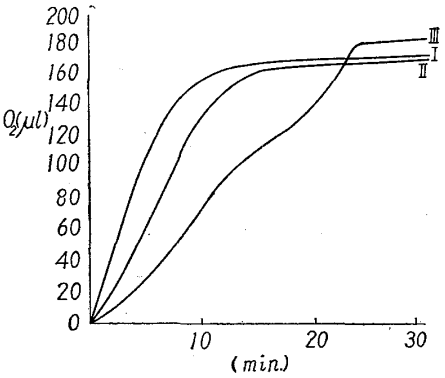


Fig. 15.

Discussion

The result indicated the steam distillate of the concentrate remained no inhibitory activity. It was clear from the results of experiment IV, V, that the inhibitor was not volatile substance and this conclusion agreed well with part I.

(Experiment VI)

The concentrate of the dialysate of the juice was passed through the column of anion exchange resin, amberlite IRA 400, at the rate of 1 cc/min.

The inhibitory activity of the solution treated with anion exchange resin was observed.

Results

Table 16. Comparison of the inhibitory activity of the solution passed through anion-exchange resin column.

Flask No. / Composition of the reaction media	I	II	III
Ascorbic acid soln.	0.5(ml)	0.5(ml)	0.5(ml)
Buffer soln.	2.0	1.0	1.0
Concentrate		1.0	
Soln. passed through anion-exchange resin column			1.0
Enzyme soln.	0.5	0.5	0.5

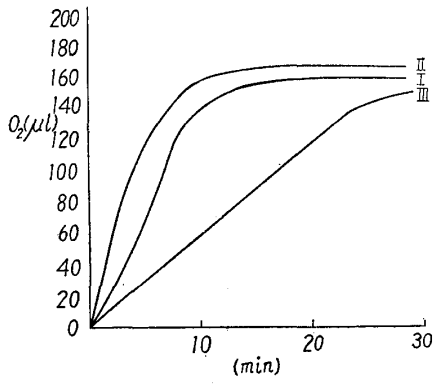


Fig. 16.

Discussion

The concentrate lost its activity after being passed through anion exchange resin column, therefore, it was determined that the inhibitor was a substance adsorbed on anion exchange resin column, namely, an organic or an inorganic anion.

(Experiment VII)

In order to determine whether the inhibitor is organic substance or inorganic, the juice squeezed by hand was concentrated *in vacuo*, then, taken in an evaporating dish and carbonized, and burnt to ashes in the muffle, about 0.3 g of ash was dissolved in 10 ml of distilled water, then, filtered with filter paper. The inhibitory activity of the filtrate was examined.

Results

Table 17. Inhibition of the ascorbic acid oxidase's action by the addition of the ash solution from mandarin oranges.

Flask No. Composition of the reaction media	I	II	III
Ascorbic acid soln.	0.5(ml)	0.5(ml)	0.5(ml)
Buffer soln.	2.0	1.0	1.0
Juice		1.0	
Ash. soln.			1.0
Enzyme soln.	0.5	0.5	0.5

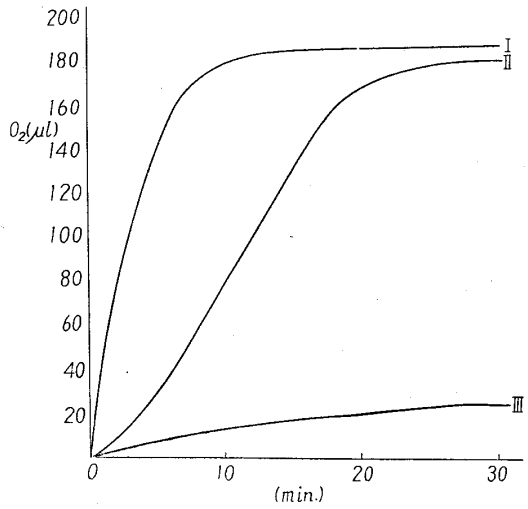


Fig. 17.

Discussion

All results in the part 2 were the same as those in the part 1.

From these results, it can be concluded that the natural inhibitor contained in summer oranges is the same contained in mandarin oranges. And it can be also concluded that the inhibitor would be a inorganic anion.

(*Experiment VIII*)

The following experiment was carried out in order to assure that the inhibitor was adsorbed on anion exchange resin.

Ash solution was passed through the column of anion exchange resin, amberlite IRA 400, at the rate of 1 cc/min. The inhibitory activity did not exist in the ash solution passed through anion-exchange resin column, of course. Enough distilled water was passed through anion exchange resin, then, 50% alcohol solution was passed through the column of anion exchange resin, to elute the adsorbed substance. But, the inhibitor adsorbed on anion exchange resin column, was not eluted, therefore, 8% NaOH solution was used to elute the inhibitor adsorbed on the anion exchange resin column. The alkaline eluate was neutralized with 5% HCl solution, concentrated to about 1/2 column of the original ash solution used for these treatments, and examined its inhibitory activity.

Results

Table 18. Inhibition of the ascorbic acid oxidase's action by the addition of the alkaline eluate of the anion-exchange resin.

Composition of the reaction media	Flask No. I	II	III	IV
Ascorbic acid soln.	0.5 (ml)	0.5 (ml)	0.5 (ml)	0.5 (ml)
Buffer soln.	2.0	1.0	1.0	1.0
Ash soln.		1.0		
Ash soln. passed through anion-exchange resin column			1.0	
Eluate				1.0
Enzyme soln.	0.5	0.5	0.5	0.5

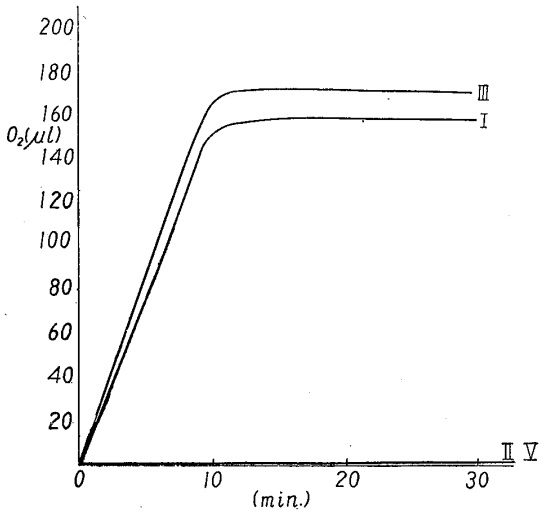


Fig. 18.

Discussion

It is clear from the results, that ash solntion passed through the anion exchange resin column, had lost its inhibitory activity, but this activity adsorbed on anion exchange resin column was recovered by the 8% NaOH solution.

So it is clarified that the inhibitor is the substance of inorganic anion.

(*Experiment IX*)

That the inhibitor would be one of inorganic anions, has become clear, so it is necessary to analyze what inorganic anion inhibitor is.

In order to analyze inorganic anions, the ash solution was passed through the column of cation-exchange resin, amberlite IRC 50, and the cation which interfered analysis of anions was eluded. Then, inhibitory activity of the solution was examined. It was supposed that inhibitory activity remained in it.

Results

Table 19. Comparison of the inhibitory activity of the ash solution with the one passed through cation-exchange resin column.

Flask No.	I	II	III
Composition of the reaction media			
Ascorbic acid soln.	0.5(ml)	0.5(ml)	0.5(ml)
Buffer soln.	2.0	1.0	1.0
Ash soln.		1.0	
Ash soln. passed through cation-exchange resin column			1.0
Enzyme soln.	0.5	0.0	0.5

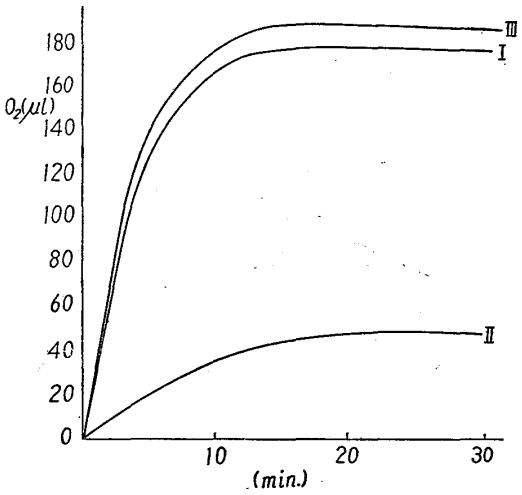


Fig. 19.

Discussion

If the inhibitor is a inorganic anion, inhibitory activity must remain in the eluate of cation-exchange resin. But the result, shown in Fig. 19, showed the ash solution passed through anion exchange resin column had lost its inhibitory activity entirely. Several trials showed the same results, and it was certified that this was not due to some erroneous treatments.

At this result was not owed to the contamination of some other anion exchange resins, and also cation-exchange resin used in this acted normally, it would be considered that the inhibitor is not a inorganic anion but the substance of the both polarity.

(*Experiment X*)

The same experiment as cited in previous part was repeated several times but the results were same, so ash solution was passed through the column of amberlite IRC 50. Then, enough distilled water was passed, and 50% alcohol was passed to elute the inhibitor adsorbed on amberlite IRC 50. But inhibitory activity was not eluted with 50% alcohol solution, therefore, 5% HCl solution was used to elute the substance adsorbed on anion-exchange resin.

The eluate was neutralized with NaOH solution and concentrated about the half volume of ash solution used, and examined its inhibitory activity.

Results

Table 20. Inhibition of the ascorbic acid oxidase's action by the addition of the HCl-eluate of cation-exchange resin.

Flask No.	I	II	III
Composition of the reaction media			
Ascorbic acid soln.	0.5(ml)	0.5(ml)	0.5(ml)
Buffer soln.	2.0	1.0	1.0
Ash soln.		1.0	
5% Hcl eluate			1.0
Enzyme soln.	0.5	0.5	0.5

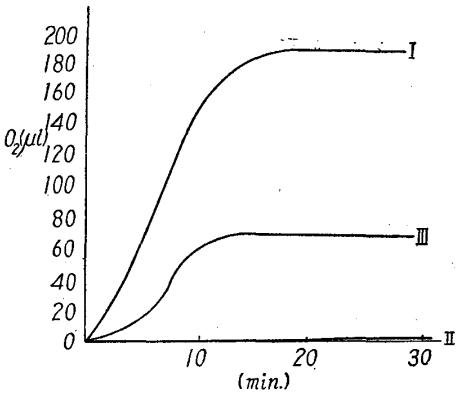


Fig. 20.

Discussion

The inhibitor adsorbed on cation-exchange resin, amberlite IRC 50 was eluted with 5% HCl solution. So it has become clear that the inhibitor is a inorganic compound of both polarity.

It is considered that the natural inhibitors against ascorbic acid oxidase, contained in oranges are two at least, one is the substance readily oxidized and the other is the inorganic compound of both polarity. And, it has not been investigated that whether organic compound which is stable for oxidation come into the mechanism of inhibitition or not. So it is necessary to examine on this point. And it is also necessary to examine the nature of this compound, if this would be present in it.

(*Experiment XI*)

It was tried that the extraction of the concentrate of the dialysate of orange juice, with chloroform and ether. As the stock period of oranges was so long at the time at which these experiments were done, the clear difference of oxygen absorption between that of the reaction media added juice and that without juice would not be observed. Then, lemons were used in this experiment.

The concentrate of the dialysate of lemon juice obtained like mandarin oranges was several times extracted with ether. And the inhibitory activity of this ether extract was examined after the evaporation of ether *in vacuo*.

Results

Table 21. Inhibition of the ascorbic acid oxidase's action by the addition of the residue of there extract.

Composition of the reaction media	Flask No. I	II	III	IV
Ascorbic acid soln.	0.5 (ml)	0.5 (ml)	0.5 (ml)	0.5 (ml)
Buffer soln.	2.0	1.0	1.0	1.0
Concentrate of the dialysate		1.0		
Ether extract			1.0	
Extract residue				1.0
Enzyme soln.	0.5	0.5	0.5	0.5

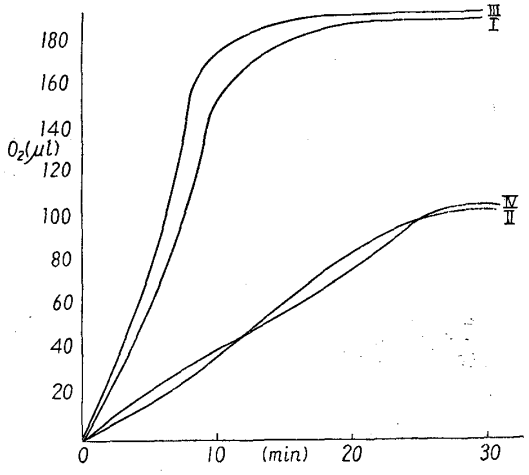


Fig. 21.

The same procedure was done with chlroform, and the inhibitory activity was also examined.

Results

Table 22. Inhibition of the ascorbic acid oxidase's action by the addition of the residue of chlroform extract.

Composition of the reaction media	Flask No. I	II	III	IV
Ascorbic acid soln.	0.5 (ml)	0.5 (ml)	0.5 (ml)	0.5 (ml)
Buffer soln.	2.0	1.0	1.0	1.0
Concentrate of the dialysate		1.0		
Chlroform extract			1.0	
Chlroform residue				1.0
Enzyme soln.	0.5	0.5	0.5	0.5

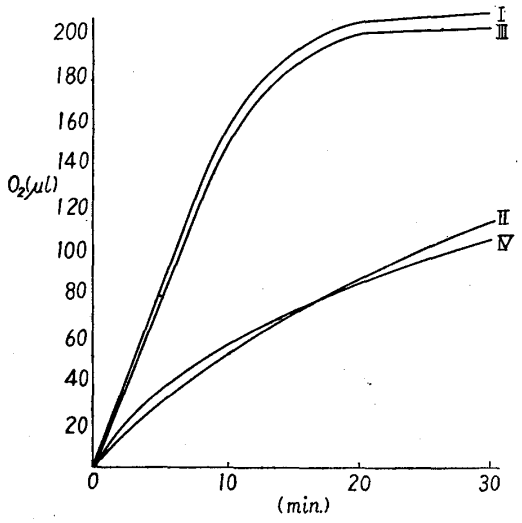


Fig. 22.

Discussion

It is clear from these results, the inhibitor is not extracted with ether and chlroform, therefore, it would be concluded that orangic compound which is stable for oxidation would not be concerned in the mechanism of the inhibition.

The authors[6] already has investigated that protein works as inhibitor against ascorbic acid oxidase but from the results that the inhibitor is dialyzable, it is considered that protein would not be concerned in the mechanism of this inhibition.

And also organic compounds of relatively low molecular such as peptide would be no effect on this inhibition. And the main natural inhibitor contained in oranges is the inorganic compound of bath polarity.

To examine the inorganic compound, further, pH of ash solution was changed to extreme acidity and also to extreme alkaliety with HCl and NaOH. And the behaviors of these acidic and alkaline solution on the anion and cation resins were investigated. But the natures for ion-exchange resins were the same as those of original ash solution.

Summary

The following facts are detected in these studies on the natural inhibitor against ascorbic acid oxidase contained in oranges.

1. The addition of 0.3–0.5% gelatin to the reaction media serves as a stabilizer for the ascorbic acid oxidase, but the effect is not so important.

2. It is considered that the natural inhibitor against ascorbic acid oxidase contained in oranges are two, one would be the substance readily oxidized with contact to air, the other would be the inorganic compound of both polarity and stable for oxidation.

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(6) C. Inagaki, H. Fukuba and A. Hatai: *Unpublished.*

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