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Distribution of the Natural Inhibitors against Cucumber Ascorbic Acid Oxidase.

(Studies on the Natural Inhibitors Against Ascorbic Acid Oxidase. Part IV)

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Introduction

We have already reported that tomato, strawberry, and egg-apple juices contain the natural inhibitors for the cucumber ascorbic acid oxidase, and also reported the characters of these inhibitors. 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-apple.[(1),(2),(3)]

In this paper, the distribution of these inhibitors in vegetables and fruits is reported, and the nature of these is also reported.

Experimental

Material used for experiments—

Vegetables and fruits used for these experiments were not specially selected or cultured. Those, as fresh as possible, were purchased at a market and used as materials for these experiments. The period of these experiments was from August to September, and so the experiments are limited to fresh materials marketed at this period.

Reagents—

The preparations of $N/10$ ascorbic acid solution, buffer solution and vegetable juice were the same as that reported in the previous papers.

Procedures—

The Warburg's apparatus was used at 30°C, as reaction temperature.

Results

In the main flask of the Warburg's vessel, 0.5 ml. of $N/10$ ascorbic acid solution, adjusted pH to 5.6, 1.0 ml. of vegetable juice, and 0.5ml.

Contribution from Department of Food and Nutrition, Faculty of Home Economic, Ochanomizu University, No. 8

Table I.

Vegetables and fruits	Amounts of oxygen Absorbed in 30 mins. ($\mu l.$)	Inhibition ratio (%)
control	560	—
peach	585	0
water melon (sarcocarp)	580	0
radish	580	0
cabbage	580	0
apple	575	0
white musk melon (*Cucumis Conomen*)	570	0
sweet pepper	570	0
stone leak	560	0
water melon (pericarp)	540	3.5
reddish turnip (leaves)	527	5.9
onion	516	7.8
reddish turnip (root)	502	10.3
turnip (leaves)	500	10.7
carrot (root)	495	11.6
spinach	490	12.5
garlic	476	15.0
grape	467	16.5
turnip (root)	436	22.1
carrot (leaves)	426	23.9
kidney-beans	423	24.5
corn	414	26.1
daidai-orange	413	26.3
taro (stem)	407	27.3
myoga (*Zingiber mioga*)	392	30.0
lotus rhizome	382	31.8
fig	370	34.0
sprouted beans	354	36.8
parsley	352	37.2
trofoil	340	39.3
green soy beans	294	47.5
sweet melon	254	54.4
strawberry	244	56.4
taro (root)	244	56.4
Brussels sprouts	220	60.7
kohlrabi	220	60.7
beafsteak plant (fruit)	190	66.2
potato	185	66.8
ginger	168	70.2
pomegranate	56	90.0
celery	20	96.4
sweet potato	8	98.6
pumpkin	—	100.0
burdock	—	100.0
spikenard	—	100.0
shungiku (*Chrysanthemum coronarium*)	—	100.0
lettuce	—	100.0
egg-apple	—	100.0

of McIlvaine's citrate-phosphate buffer (pH 5.6) were pipetted, and 0.5 ml. of cucumber juice was also pipetted into the side arm. The vessel was incubated for 10 minutes in the constant water bath kept at 30°C for the sake of the temperature equilibrium, then the content of the side arm was poured into the main flask. The absorption of oxygen in 30 minutes by this reaction medium was measured at intervals of 3 minutes, and the amount of oxygen absorbed in 30 minutes was compared with that of the reaction medium without vegetable juice. The results obtained with 47 kinds of vegetable and fruit juices are listed in Table I. In these juices, those obtained from egg-apple, celery, pumpkin, burdock, spikenard, shungiku, lettuce, and sweet potato had the powerful inhibiting activity against the cucumber ascorbic acid oxidase.

As the mechanism of this inhibition by egg-apple juice was already known, the investigation as to whether the other 6 kinds of juice behave as that of egg-apple juice or not against the ascorbic acid oxidase

Table II. Compositions of the reaction media

Reaction medium I	Main flask	*N*/10 Ascorbic acid Vegetable juice Buffer solution	0.5 ml. 0.5 ml. 1.0 ml.
	Side arm	Cucumber juice (as enzyme solution)	0.5 ml.
Reaction medium II	Main flask	*N*/10 Ascorbic acid Buffer solution	0.5 ml. 1.5 ml.
	Side arm	Vegetable juice (as enzyme solution)	0.5 ml.

Table III. Amount of oxygen absorbed in 30 minutes with the reaction media I and II.
(Compositions of these media are cited in Table II.)

Vegetables	Amount of oxygen absorbed ($\mu l.$)	
	Reaction medium I	Reaction medium II
Cucumber	4*	560
Pumpkin	6	548
Celery	20	256
Spikenard	2	147
Shungiku	6	126
Lettuce	2	120
Sweet potato	8	112
Egg-apple	—	100
Burdock	—	22

* Pumpkin juice was added instead of cucumber juice as enzyme solution

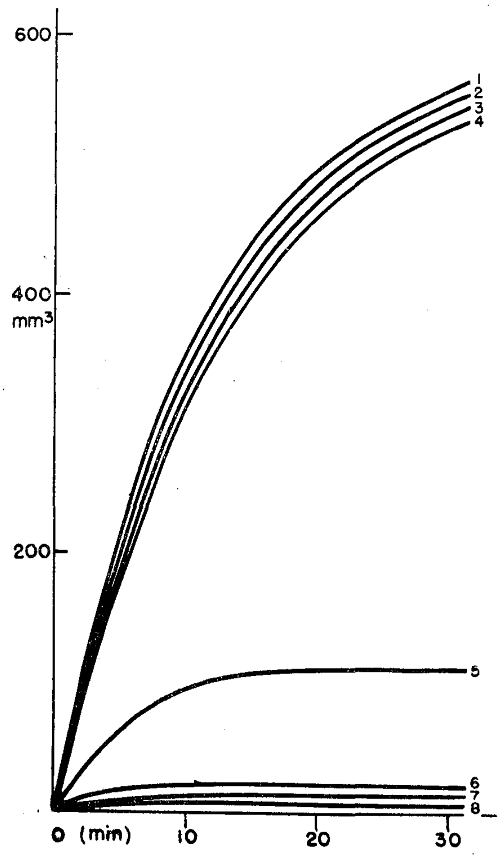

Fig. 1. Inhibiting actions of the sweet potato juice and the burdock juice against the cucumber ascorbic acid oxidase.

Reaction medium I
1. Steam distillate of sweet potato juice
2. Steam distillate of burdock juice
3. Sweet potato juice boiled for 10 mins.
4. Burdock juice boiled for 10 mins.
7. Sweet potato juice
8. Burdock juice

Reaction medium II
5. Sweet potato juice
6. Burdock juice

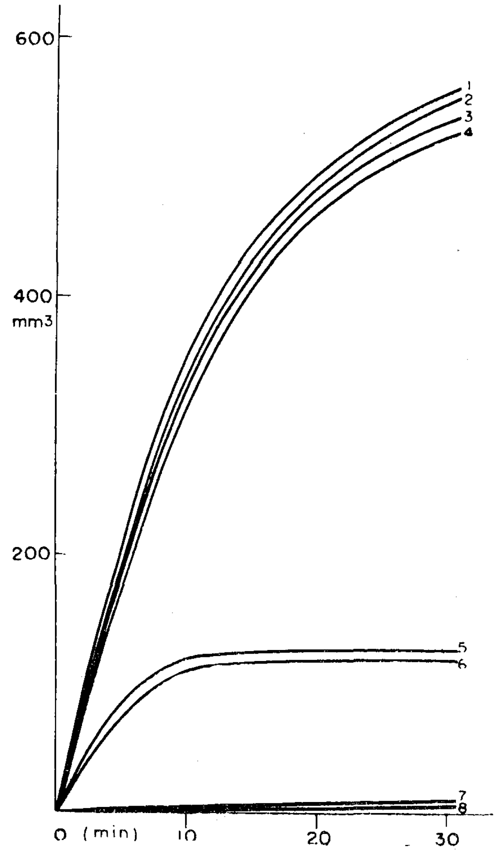

Fig. 2. Inhibiting actions of the shungiku juice and the lettuce juice for the cucumber ascorbic acid oxidase.

Reaction medium I
1. Steam distillate of shungiku juice
2. Steam distillate of lettuce juice
3. Shungiku juice boiled for 10 mins.
4. Lettuce juice boiled for 10 mins.
7. Shungiku juice
8. Lettuce juice

Reaction medium II
5. Shungiku juice
6. Lettuce juice

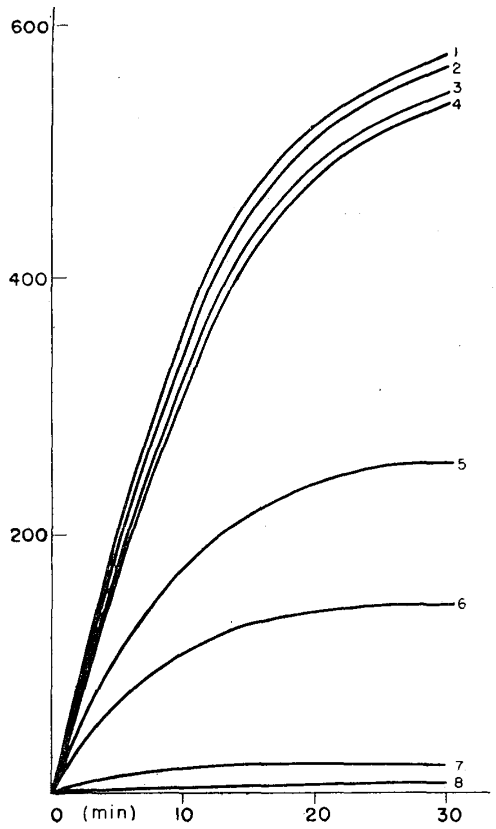

Fig. 3. Inhibiting actions of the celery juice and the spikenard juice against the cucumber ascorbic acid oxidase.

Reaction medium I
1. Steam distillate of celery juice
2. Steam distillate of spikenard juice
3. Celery juice boiled for 10 mins.
4. Spikenard juice boiled for 10 mins.
7. Celery juice
8. Spikenard juice

Reaction medium II
5. Celery juice
6. Spikenard juice

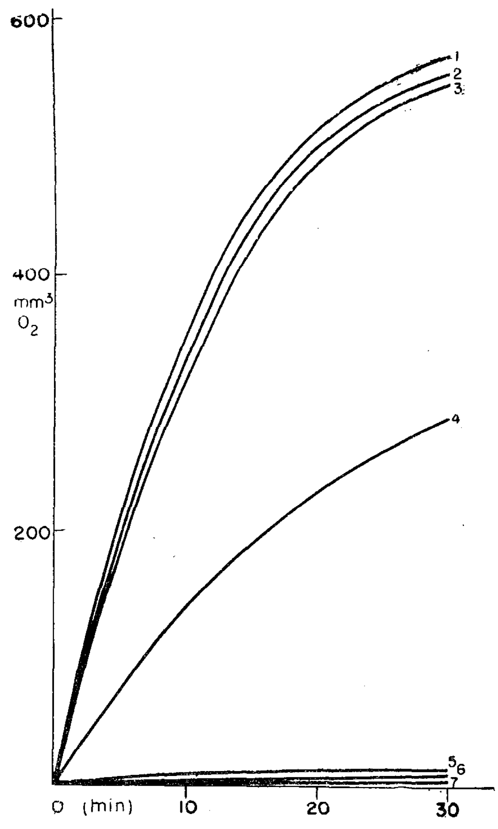

Fig. 4. Inhibiting actions of the pumpkın juice against the cucumber ascorbic acid oxidase and the changes of this activity by the various treatments.

Reaction medium I
2. Steam distillate of pumpkin juice
3. Pumpkin juice boiled for 10 mins.
6. Pumpkin juice

Reaction medium II
1. Cucumber juice
4. Pumpkin juice
5. Pumpkin juice boiled for 10 mins.
7. Oxygen absorption curve owing to the autoxidation of ascorbic acid.
(Composition of the reaction medium: $N/10$ ascorbic acid 0.5 ml. and buffer solution 2.0 ml.)

was further pursued. The inhibiting activities of these juices, the boiled juices which had been prepared by boiling the fresh juice for 10 minutes, and the steam distillates of these juices were determined with the reaction media having the compositions cited in Table II. The amount of oxygen absorbed in 30 minutes by these reaction media is listed in Table III, and the oxygen absorption curves by these reaction media and also by those obtained with the media to which was added the boiled juice or the steam distillate instead of the vegetable juice in 'Reaction medium I' cited in Table II are illustrated in Figs. 1 to 4.

From these results, it was possible to infer that these 8 kinds of juice had some ascorbic acid oxidase's activity, while these reacted inversely as inhibitor for the enzyme with the 'Reaction medium II' cited in Table II. In order to know the mechanism of this inversion, the amount of oxygen absorbed with the 'Reaction medium I was measured, in which a half of cucumber juice pipetted into the side arm was employed as enzyme solution, while the remains pipetted into the main flask was used as the vegetable juice like the other 47 kinds. The variation of the oxygen absorption by the further addition of oxidase solution to the 'Reaction medium II' 10 minutes after the start of the ascorbic acid oxidase's action was also measured by the use of the Warburg's vessel with two side arms attached to it, and these results are illustrated in Fig. 5.

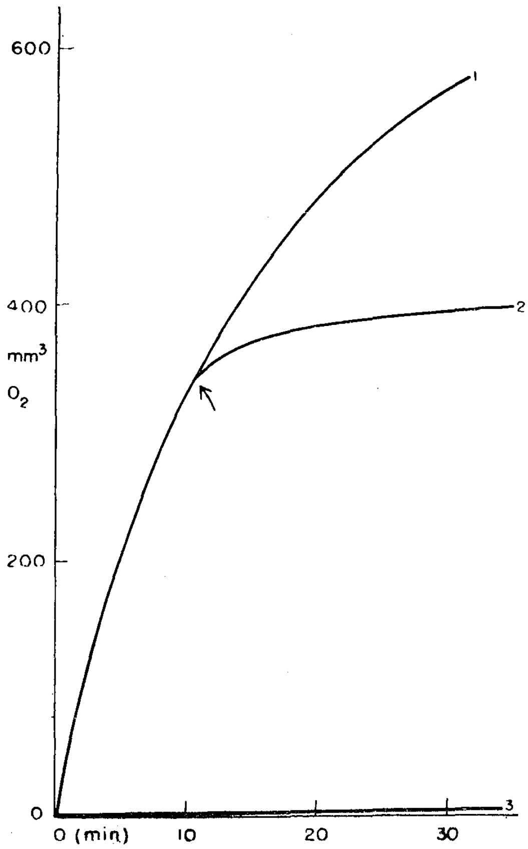

Fig. 5. Influence of the addition of cucumber juice to the ascorbic acid oxidase's reaction medium.

1. Cucumber juice as enzyme solution only (The composition of the reaction medium is like that of 'Reaction medium II')
2. Inhibition of ascorbic acid oxidase's reaction of cucumber juice by the further addition of cucumber juice. The vessel with two side arms attached to it is used. The reaction medium is composed of $N/10$ ascorbic acid 0.5 ml., buffer solution 1.0 ml., and cucumber juice 1.0 ml. (Each 0.5 ml. of juice is pipetted into the side arms and the one is poured into the flask to start the reaction, while the other is poured 10 minutes later.
3. Inhibition of cucumber ascorbic acid oxidase's action by the addition of cucumber juice to the reaction medium. The composition of the reaction medium is the same as that of 'Reaction medium I' cited in Table II. A half of cucumber juice is added in the flask and the remainder is added in the side arm.

Discussion

We have measured the inhibiting activity of 47 kinds of vegetable and fruit juices against the cucumber ascorbic acid oxidase and found as mentioned above, that 8 kinds of juices had the powerful activity against this enzyme. Nevertheless, we could not find the relationship between this activity and the botanical class of these vegetables and fruits.

The inhibiting activity was not the characteristic property of some kinds of juices; instead almost all kinds of juices tested had this activity, even though it was weaker.

We have reported in the previous reports that tomato juice and strawberry juice showed the powerful inhibition against this enzyme, while this time, these exhibited only a little activity, and as mentioned in the first report, the activity of each kind of vegetable will be much influenced by the maturity, the species, and so on.

We have studied the nature of these inhibitors using the boiled juice and the steam distillate of these juices. If the inhibitor is volatile organic compounds, as in the case of tomato juice, the inhibition against the ascorbic acid oxidase will not be observed in the case of the boiled juice, owing to the loss of these compounds during the boiling, and the inhibiting activity, when this is the case, will have to be observed with the steam distillate of these juices. But as shown in the experimental parts, we could not find the activity with the boiled juice and also with the steam distillate. Then it is assumed that these inhibitors are thermolabile, and perhaps protein.

From the fact that the absorption of oxygen could be observed when these juices were added in the medium composed of ascorbic acid solution and buffer solution, we could assume that this absorption of oxygen was due to the presence of ascorbic acid oxidase in these juices. Further, as illustrated in Fig. 4, for example, the pumpkin juice lost its activity for the oxidation of this acid by boiling, and from this, we could additionally assume that this activity was not due to the inorganic Cu ion but due to the ascorbic acid oxidase contained in this juice.

The data in Table III showed us that these 8 kinds of juice, except burdock, had themselves some activity as ascorbic acid oxidase, and when these juices were added in the media having the composition given as 'Reaction medium II' in Table II, these juices did not react as enzyme but inversely react as inhibitor. Then the inhibitor which was thermolabile was not the true inhibitor having the low molecular weight, but only an apparent one. The inhibiting action was assumed only to have resulted from the inversion of one kind of ascorbic acid oxidase to the inhibitor for the other kind of ascorbic acid oxidase. This inversion was a strange and interesting phenomenon.

To ascertain this assumption, the change of oxygen absorption by the addition of cucumber juice to the reaction medium which contained the cucumber juice, as enzyme solution was measured. As shown in Fig. 5, Curve 3, we could observe no absorption, but in this case, owing to the powerful activity of cucumber juice as ascorbic acid oxidase, the ascorbic acid added in the medium might be consumed up during the preparation of the reaction medium and the temperature equilibrium of this medium, and then what we have determined might be no more than the apparent inhibition. To avoid this trouble, the flask attached two side arms was employed, and the cucumber juice was pipetted into these two arms, the contents of these were poured into the main flask with the interval of 10 minutes. The results of these experiments cited in Fig. 5. In this experiment, we could observe some inhibition compared with the one carried out with the medium given in Table II, 'Reaction medium II'. From these results, the inversion-reaction as the mechanism of the inhibition was further ascertained.

Then let us further study the mechanism of this inversion. As shown in the previous paper, the egg-apple juice exhibits an inhibiting reaction by the combination of the enzyme and Cu ion, and this enzyme itself has Cu ion as the prothetic group of ascorbic acid oxidase; therefore, the prothetic group of one kind of ascorbic acid oxidase will combine with the prothetic group of the other kind, namely, the Cu–Cu combination will be formed and by this combination, the inhibition will be performed. This combination will be promoted by the affinity present between the different kinds of protein, and the case of cucumber-cucumber reaction, the cucumber juice previously added in the reaction medium react as oxidase on the ascorbic acid, and during the reaction the oxidase itself is denaturated and loses its activity. This denaturated protein reacts on the fresh cucumber juice as a different kind of protein and then the affinity between these two supports the combination of the two.

In Table I, we could find a little inhibiting activity with the pericarp juice of sweet melon, while this juice itself had a powerful ascorbic acid oxidase's action and the amount of oxygen absorbed in 30 minutes with the 'Reaction medium II' cited in Table II reached to 524 μl. Then the inhibiting activity cited in Table I was nothing but an apparant one.

We are indebted to Misses Emiko Hayashi, Tomiko Machida and Atsumi Itoh for their cooperation with us.

Summary

The inhibiting action against the cucumber ascorbic acid oxidase was measured with 47 kinds of vegetable and fruit juices, and of these, this action with the juices obtained from egg-apple, celery, pumpkin, burdock, spikenard, shungiku, lettuce, and sweet potato was especially great.

It was ascertained that these juices, except burdock juice, had the powerful ascorbic acid oxidase's action and these when added to the media which contained the cucumber juice, showed inversely the inhibition against the cucumber ascorbic acid oxidase.

It was also assumed that this inversion was due to the combination of the prothetic group of one kind of ascorbic acid oxidase and the prothetic group of the other kind of oxidase forming the Cu–Cu linkage.

Literature

1. C. Inagaki, H. Fukuba, *Nat. Sci. Rep. Ochanomizu Univ.*, **4**, 235–241 (1954)
2. C. Inagaki, H. Fukuba and A. Matsushita, *Ibid*, **5**, 92–100 (1954)
3. C. Inagaki, H. Fukuba and A. Matsushita, *Ibid*, **5**, 313–322 (1955)

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