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Biochemical Study on Soft-rotted Sweetpotatoes

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SUMMARY

Sweetpotato infected by Soft-rot, *Rhizopus nigricans*, has been investigated for its biochemical changes. The results of the present experiment are summarized as follows:

1. From the Soft-rotted tuber, nineteen Ehrlich's positive substances and three fluorescent compounds which had not been found in a healthy tuber were detected with thin layer chromatography.

2. Ipomeamarone and umbelliferone, each of which are predominating substances among the Ehrlich's positive and fluorescent compounds, were isolated by silica gel column chromatography and studied for their identities.

3. Time course biosynthesis of the metabolites was studied and discussed for their possible biosynthetic pathways.

4. Among the metabolites, ipomeamarone and ipomeamarone-like substances have enzyme inhibitive action against α-amylase and probably other enzymes; the inhibition type was determined as the uncompetitive one.

5. Ipomeamarone obtained from soft-rotted sweetpotato was proved to have an uncoupling action as 2,4-dinitrophenol.

I. INTRODUCTION

Sweetpotato (*Ipomea batatas*), which is utilized as food and feed stuffs, and industrial raw material for the manufacture of starch and alcohol, has been cultivated widely in the temperate and tropical areas. Sweetpotato production in Korea has been increasing year by year for the main reason that its starch yield per unit area is superior to other crops. However, sweetpotato which originated from tropical area ass a perennials are very liable to cold injury due to the compulsory adaptation to a higher altitude area as Korea to an annual crop, or due to their critical chilling temperature of 9°C. Harvest in this area begins from early October to mid November. During this period, temperature changes around the critical point have been recorded. Cold injury comes mainly after harvest on the field and during storage, always bringing nearly the typical disease, soft-rot, caused by a mold, *Rhizopus nigricans*.[1-3] After being injured by chilling, an infection of soft-rot fungus follows; within a week, the tuber is softened to a serious state which is worse than useless. At least nine species of *Rhizopus*, with *R. nigricans* (*stolonifer*) predominating, are implicated in the etiology of soft-rot, which is reported to be the most destructive post-harvest disease.

What are the biochemical changes in sweetpotato infected by soft-rot? It is desirable to solve this problem for both the biochemical interest and the

industrial aspects. Although considerable attention has been paid to soft-rot in the pathological and microbiological fields,[5-12] nothing is known of its biochemical changes, except a few classical reports[13,14].

A series biochemical and pathological study have been carried out on the black-rotted sweetpotato tubers caused by *Ceratocystis fimbriata*. Isolation of ipomeamarone, an abnormal metabolite, and its biochemical studies were reported[15-19]. The metabolites include ipomeamarone and ipomeamarone-like terpenoids, polyphenols including chlorogenic acid, iso-, neochlorogenic acids[21,22] and coumarins such as umbelliferon, scopoletin and esculetin.[23-25] Uritani and Akazawa proposed the following biosynthetic pathway of ipomeamarone;[26,27]

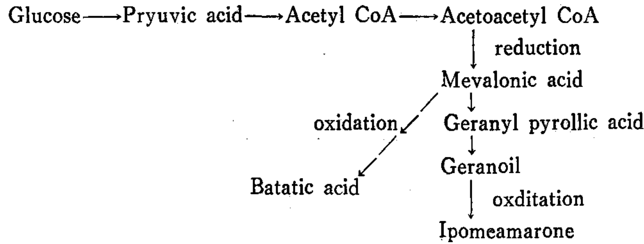


Fig. 1. Proposed Ipomeamarone Biosynthetic Pathway

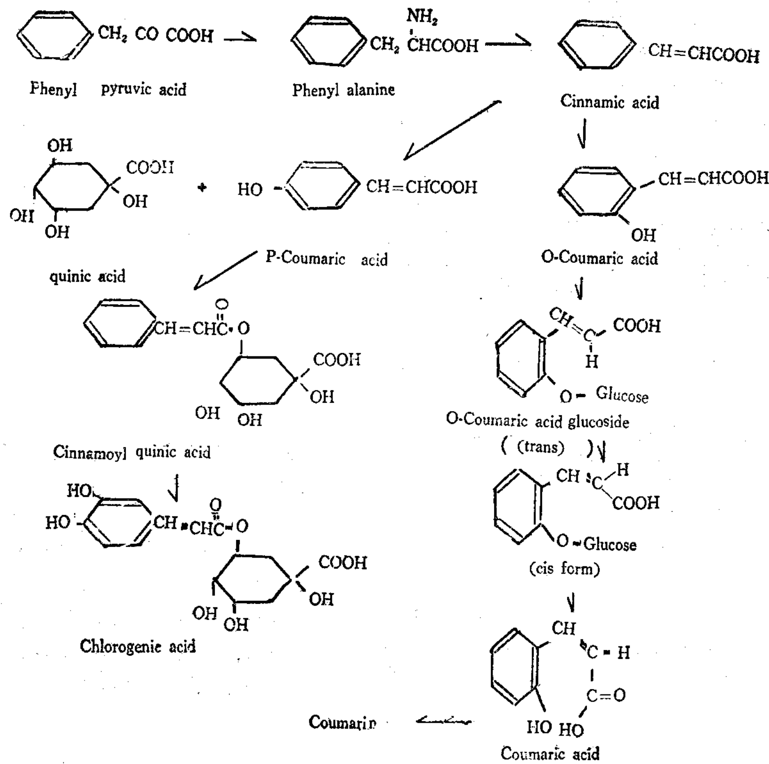


Fig 2. Proposed Biosynthetic Pathway of Polyphenol and Coumarin Compounds

Polyphenol biosynthesis in the infected tuber has been understood to be the same as that within the healthy tuber proposed by Neish[28] and Koukol et. al.[29] as shown in figure 2. These schemes are generally accepted in black-rotted sweetpotato tubers by the isolation of intermediates of the pathway and radioisotope incoorporation experiment.[27]

Soft-rot is as well known as a destructive disease

in sweetpotato as black-rot. While the latter has been extensively investigated as previously mentioned, the first, however, still remained without any appreciable biochemical study. Between the two rots, several differences can easily be summarized as follows:[1] points; infection mechanism—soft-rot infection is usually possible only after harvest and especially after a chilling injury, but black-rot easily attacks tubers both in the field and after harvest, or during storage and no pre-conditions, except mechanical injury, are requested to induce the infection.[2] States of tuber diseased—the typical symptom of softening, which is caused by the strong pectolytic enzymes from the responsible organism, can not be found in black-rot at all.[3] Color changes due to decay—tissues infected with soft-rot have no changes of color even after complete decay in contrast to the typical black spots on the tubers attacked by black-rot. The above differences requested the author to attempt this biochemical study of soft-rotted sweetpotato. Prime interest was originally posed on whether any biochemical changes, comparable to those of black-rot are possible and what the differences in the biochemical aspects if any. An approach made in the course of experiment is reported here.

II. MATERIALS AND METHODS

1) Sweetpotato:

A variety of sweetpotato, Choong-Seung(Okinawa) No. 100 harvested in Suwon in mid of October, 1967, were cured and stored at 13°C, a recommended storage temperature.

2) Soft-rot Fungus:

Rhizopus nigricans isolated from a soft-rotted tuber used for the inoculation, and the organism was incubated on slice of fresh sweetpotato in Petri-dish was kept in a refregerator to maintain pathogenicity.

3) Preparation of Soft-rotted Sweetpotato:[12]

Inoculation of *R. nigricans* into sweetpotato tuber was made as follows. Infections were made by inoculating spores of the organism into wounds which were made by striking the tuber with the edge of a knife, and charring the tuber with a flame. Spores were placed on charred wounds 0.1-0.5 cm deep made by repeatedly striking the tuber with the blunt edge of a knife. Inoculated tubers were incubated at 20-30°C and 95-100% of relative humidity for one or two weeks. Completely rotted tubers were collected from the lot.

4) Preparation of Crude Alcohol Extract:

Five grams of infected tissue were homogenized with 30 ml of 95% methanol by the Potter-Elvehjem glass homogenizer and the resulting extract was centrifuged to obtain the supernatant, or methanol extract.

5) Extraction and Distillation:

The overal extraction and distillation procedure are shown schematically in figure 3. Dried powder of soft-rotted sweetpotato were added with the same weight of 80% ethanol and the mixture was refluxed in hot water bath for thirty minutes, and the residue was further extracted for ten minutes with the same volume of 80% ethanol. After cooling the mixture alcohol extract was obtained by centrifugation and the extract was concentrated in a rotary evaporator near to syrup. The concentrate was again extracted with ethylether in a Soxhlet type liquid-liquid extractor with occasional shaking. The ethereal extract was washed with 5% sodium carbonate to remove acidic materials and dried overnight with anhydrous sodium sulfate. After evaporation of ether, the remaining dark brown oily crude material was subjected to vacuum distillation at 125°C/4.0 mm Hg for the isolation of Ehrlich's positive[37] substances.

6) Isolation and Purification of Ehrlich's positive Substances:

Separation and purification of the compounds showing positive reaction against Ehrlich's reagent made by the repeated conventional silica gel column chromatography.[20] The column was packed by gravity with a slurry of silica gel (Mallinckrodt, chromatograhic grade, 100 mesh), 260 g of which was suspended in n-hexane. About two grams of oily material dissolved in a small volume of n-hexane were applied to the column. A device was constructed for a linear gradient elution, consisting of a reservoir and a mixing vessel of identical shape (round bottom flask), connecting with a syphon of 2 mm glass capillary tubing between two vessels and a similar tube led from mixing vessel

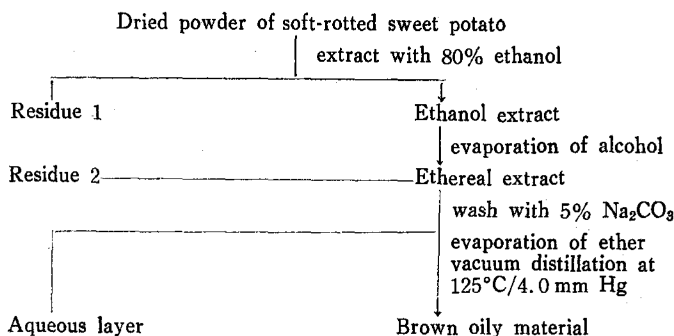


Fig. 3. Extraction and Distillation Scheme

to chromatographic column. Ethylacetate in n-hexane was the solvent system of the elution. The concentration of ethylacetate in n-hexane is linearly increased by the following equation:[32]

$$(El) = M_2 - (M_2 - M_1)\left(1 - \frac{\phi}{VR}\right)$$

where El: concentration of ethylacetate in n-hexane

M_1: initial ethylacetate concentration in mixing vessel

M_2: ethylacetate concentration in reservoir

ϕ: elution volume

VR: liquid volume of reservoir

The composition of resultant effluents collected with an automatic fraction collector was examined by simultanenous running of chromatographic technique which will be mentioned later. Collections of the corresponding fraction to thin layer chromatograph (TLC) were extracted with hot methanol and evaporated the solvent, and the condensed material was further purified by repeated running of the same chromatographic technique. Purification by paper chromatography with Toyo No. 51 paper was also made.

7) Isolation and Purification of Fuorescent Substances:

Aqueous layer (Residue 2, in Fig. 3) resulted from the washing of ethereal extract was shaken vigorously with a half volume of chloroform after adjusting pH to 6.4 with 3N H_2SO_4 and the extraction with chloroform was repeated 5 times. The combined chloroform extract was evaporated at 30°C to a small volume in a rotary evaporator attached with oxygen absorbent alkaline pyrogallol solution, and the condensed material was subjected to silica gel column (2×20 cm), which was embeded with slurry of silica gel suspended its fourty grams, which was previously hydrated with 15 ml of water, into chloroform, and stepwisely eluted with chloroform and 10% ethylacetate in chloroform. The resulatant fluorescent fractions were collected with fraction collector. Purification was made by repeated running of the same column chromatograph. For further purification of the compound, collected effluent corresponding to fluorescent spot on the chromatostrip was concentrated near to dryness at 30°C. Attention was paid to avoid oxidation during the evaporation. Samll amount of methanol was added to dissolve the compound and applied to TLC plate (20×20 cm, 0.5 mm thickness). After development with 25% ethylacetate in n-hexane the fluorescent bands were scratched and extracted with warm ethanol. The supernatant by obtained centrifugation was allowed to stand overnight in a refregerator to be crystalized. Recrystalization was made in ethanol solution. Figure 4 shows the schematical isolation and purification procedure.

8) Preparation of Silica Gel Chromatostrip:

Basic preparation procedure of chromatostrip was essentially the same as reported elsewhere.[20] Ten grams of silica gel (E. Merk, thin layer chromatographic grade, 10-40 μ of its grain size) and equal amount of water were mixed throughly, and the slurry of the gel was rapidly spreaded over glass plate (5×20 cm for the strip, 20×20 cm for the plate) as thin layer and dried for one hour in an electrical oven, the desirable temperature being 90°C.

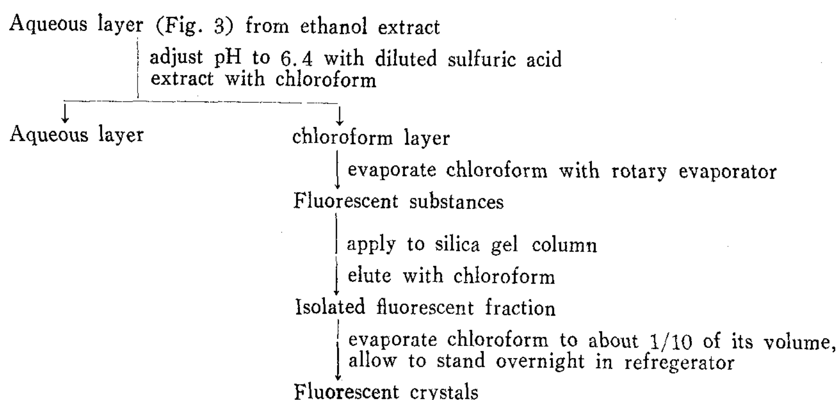


Fig. 4. Schematical Isolation and Purification Procedure

9) Paper Chromatography:

The conventional ascending paper chromatographic techniques were developed with Whatman No. 1 paper for both Ehrlich's positive substance and fluorescent compounds. Methanol, butyl acetate and butanol : acetic acid : water (9 : 1 : 1) for Ehrlich's positive substance, and 5% acetic acid, water saturated butanol, butanol: acetic acid: water (4 : 1 : 1) and ethano: NH_4OH: water (80 : 5 : 15) for fluorescent compound were used.

10) Reactions:

Several color reactions were performed for the identity of the compounds.

a) Ehrlich's reaction:[31] The reagent was prepared by dissolving 5.0 g of p-dimethylaminobenzylaldehyde (Matheson Coleman and Bell) in 50 ml of each of concentrated hydrochloric acid and 95% ethanol, and the color was developed by spraying the reagent on paper or chromatostrip and heating at 100°C.

b) Liebermann's reaction:[33] Ten drops of acetic anhydride and a few drops of concentrated sulfuric acid were added to one or two drops of purified compound dissolved in 2 ml of chloroform.

c) Zeisel's reaction: Methoxy group test were carried out by the reaction as described elsewhere.[36]

11) Ultra Violet Spectra:

Ultra violet absortion spectra of the compound in 95% ethanol solution were measured by Beckman DU spectrophotometer.

12) Infrared Spectra:

Infrared absorption spectra of the purified in KBr plate were measured by Perkin-Elmer Infrared spectrophotometer.

13) Analytical Method:

1. Analysis of Ipomeamarone

a) Preparation of ethanol extract: Thirty grams of sweet potato slice were inoculated with spore suspension of *R. nigricans* on the mid of the slice and incubated at 23°C in a Petridish embeded with wet cotton on the bottom. At 24 hours intervals, sweetpotato slices were taken out for impomeamarone analysis. The infected tissue were blended in 95% ethanol solution with Waring blendor for five minutes and washed with 50 ml of ethanol solution. The mixture was centrifuged and supernatant alcohol extract was concentrated near to syrup. The resultant syrup was filled up to 10 ml with carborlyl free methanol.[35] A 1 ml of aliquot was applied to thin layer chromatostrip and developed with 10% ethylacetate in n-hexane. To fine out the exact localization of ipomeamarone on the strip, margines of the strip were sprayed with Ehrlich's reagent. The coresponding portion of silica gel was scratched with a spatula and extracted with 5 ml of 95% carbonyl free methanol. Its 0.1 ml was subjected to carbonyl determination.

b) Spectrophotometric Analysis: Carbonyldetermination in ipomeamarone structure was followed by the method of Lappin and Clark.[35] One ml of carbonyl free methanol saturated with 2.4-dinitrophenylhydrazine (Eastman Organic Chemicals) and 0.02 ml of concentrated hydrochloric acid were added to above eluate and incubated at 50°C for 90 minutes. Reaction mixture was taken out and

cooled in water. By adding 5 ml of 10% KOH solution in 50% carbonyl free methanol, brown color was developed and after 10 minutes its color intensity was measured, at 470 mμ with Beckman DU spectrophotometer. The standard curve was constructed with a chromatographically purified impomeamarone. The linearity between absorbancy and concentration of impomeamarone holds up to 400 μg/ml(Fig. 5).

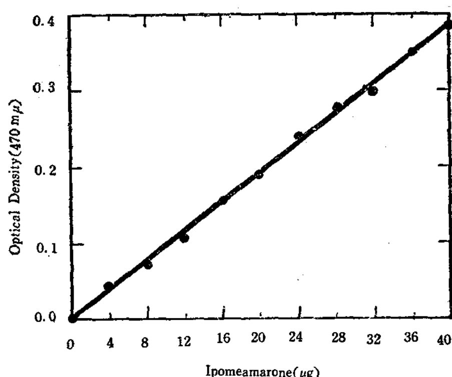


Fig. 5. Standard Curve of Ipomeamarone

2. Analysis of Umbelliferone

A 0.1 ml of ethanol extracts prepared as previously mentioned at ipomeamarone analysis in this text 13-1, was quantitatively applied to a silica gel chromatostrip and developed in ethylacetate: n-hexane (25 : 75). The fluorescent band detected under UV-lamp was scratched with a spatula from the strip and eluted by a 5 ml of hot methanol. The blank zone corresponding to the band was treated in the same manner and the resulting eluate served as a control. Quantitative estimation of umbelliferone, newly established by the author, was made by measuring the absorbancy at 320 mμ with Beckman DU spectrophotometer. The standard curve of umbelliferone was made of authentic one (K and K Lab. Co.) The linearity between absorbancy and concentration of umbelliferone holds up to 300 μg per mililiter of ethanol (Fig. 6).

14) Methods in Enzyme Inhibition

a) Enzyme: For the study of enzymatic inhibition of impomeamarone, α-amylase (Nutritional Biochemicals Cooperation) was used as a model

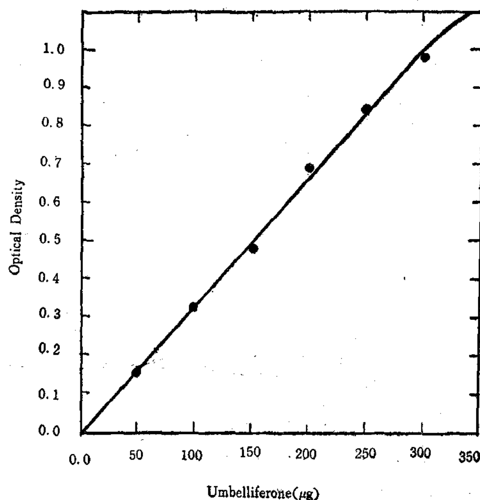


Fig. 6. Standard Curve of Umbelliferone

enzyme. The enzyme solution was prepared by dissolving 10 mg of enzyme powder in 100 ml of 0.4 M acetate buffer (pH 4.8-5.0).

b) Substrate: Various amount of starch (potato starch) were dissolved in 4 ml of lN NaOH solution and diluted about to 80 ml of water after standing over night. The solution was adjusted to pH 4.8 with lM acetic acid solution and made volume to 100 ml.

c) Inhibitor: Purified ipomeamarone was served as an inhibitor. Ihibitor solution was prepared by dissolving 20 mg of ipomeamarone in 10 ml of 50 % ethanol solution. Its concentration was adjusted by diluting with 50% ethanol.

d) Reaction procedure: To 4 ml of substrate in a test tube (18×18 cm), 1 ml of the inhibitor solution was added. After incubation in a water bath at 40°C for ten minutes, 1 ml of enzyme solution was added and incubated for 20 minutes. The reaction was blocked by adding 5 ml of lM acetic acid.

e) Enzyme assay: Two mlilater of reaction mixture were poured into 10 ml of 0.005% I_2 solution and the resulting blue color was measured spectrophotometically at 690 mμ with Colemann activity spectrophotometer. Caculation of enzyme Universal was followed by Blue value method[36] after calbriation being made.

15) Manometric Measurement of Oxygen Uptake by Yeast

a) Yeast: *Saccharomyces cerevisiae* was cultured in Kojic extract at 27°C for 24 hours and 0.5 g of its resting cell, washed with the following basal medium, were suspended in 100 ml of the medium.

b) Basal medium: Basal medium was consisted of 0.05 M acetic acid, 0.05 M sodium acetate, 2.4×10^{-3}M $MgSO_4\cdot7H_2O$, 1×10^{-3}M $MnSO_4\cdot H_2O$, 1×10^{-4}M thiamine chloride and 0.02 M KH_2PO_4.

c) Impomeamarone and 2.4-dinitrophenol(DNP) solution: 8×10^{-3}M ipomeamarone solution was prepared as described at 14-C, in this text. To examine the effect of DNP (Fisher Chemical Co.) on respiration, 2.5×10^{-4}M DNP solution was prepared and added in place of ipomeamarone.

d) Procedure of Manometric Measurement:[37] Oxygen uptake by yeast was measured by Warburg technique. One mililiter of yeast suspension and 2 ml of basal medium were placed in the main compartment. A 0.3 ml of 0.3 M glucose and 0.5 ml of 8×10^{-3}M ipomeamarone solution were also placed in the side arms. Incubation was performed at 30°C with 0.2 ml of 10% KOH as carbon dioxide absorbent in the center wall. After a 5-minute equilibration period, readings were made every 5 minutes over a 30-minute period, and corrected for any changes in the thermobarometer from its zero time reading.

III. EXPERIMENTAL RESULTS

1)Alcohol Extract of Soft-rotted Sweetpotato

After incubation at 23-25°C for one week, the tissue of tuber was completely softened (Fig. 7). The alcohol extract (see II, 4) obtained from the

Fig. 7. Sweetpotato tubers:
a: healthy b: decayed

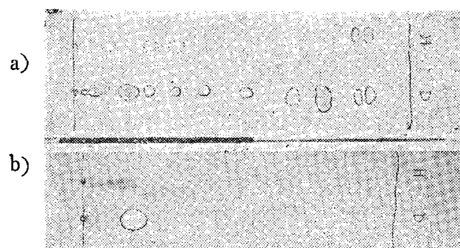


Fig. 8. Thin Layer Chromatograms of Alcohol Extract

a: after spray of Erhlich's reagent.
b: under UV-light. H and D denote healthy and decayed tubers, respectively.

decayed tuber was compared chromatographically with that of healthy one, or thin layer chromatograms showed that new substances were produced in the case of soft-rot decay, as shown in figure 8.

2) Isolation and Purification of an Ehrlich's positive Substance

Among the several substances showing positive reaction with Ehrlich's reagent, a dominant spot, pink red color but liable to change to grey-blue immediately after color development on thin layer chromatogram, was observed. Vacuum distillation, mentioned elsewhere in this paper on II-5, was so effective for isolation that the number of the spots was not changed but the changes in relative concentration of the Erhlich's positive substances was appreciably affected. The main spot, supposed to be ipomeamarone reported by Uritani[30] was appeared more condensly on thin layer chromatogstrip as shown in figure 9.

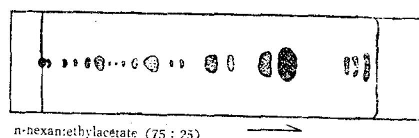


Fig. 9. Thin Layer Chromatogram of Vacuum Distillate. Cross hatched circle is condensed and pink red spot.

A 12 g of distillate was yielded from 4 kg of soft-rotted sweetpotato powder. The oily and oderous distillate was subjected to silica gel column chromatography for the separation of the main

spot. Minor spot having similar behavior on the chromatostrip and in color reaction, was very easily contaminated, a clear separation, however, was attained from repeated running of chromatography with gradient elution instead of stepwise system in the operation of column chromatography (Fig. 10). Column chromatographically purified main fraction has a nearly colorless and typical odor.

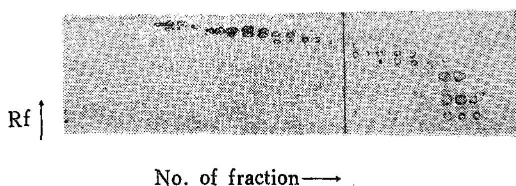


Fig. 10. Simultaneous TLC of Fractions from Column Chromatostrip.

After evaporation of hexane from the purified fraction, 2.6 grams of ipomeamarone were yielded.

3) Isolation and Purification of Fluorescent Substances from Soft-rotted Sweetpotato.

A 5.3 g. of condensed fluorescent substances was obtained after evaporation of chlorofrom layer (Fig. 4) under reduced pressure passing N_2 gas. The condensed fraction contained one main and two minor fluorescent spots as shown in figure 11. The intensity of the main fluorescent spot on the TLC was as much ten times as the other minor spots, which were not detected in crude alcohol extract (Fig. 8).

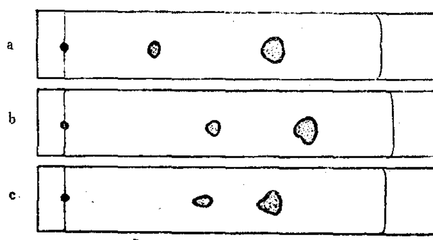


Fig. 11. TLC of Fluorescent Substances in various solvent systems.

a) Toluene: Ethylacetate:Formic acid(74 : 25 : 1)
b) Benzene: Ethylacetate(75 : 25)
c) n-Hexane: Ethylacetate : Formic acid(75 : 25 : 1).

The condensed one was applied to silica gel column chromatography and three fluorescent fractions were clearly separated, into two fast moving fractions F-1, F-2 and S, that of slow moving with chloroform as the solvent system. These isolates were purified by repeating of silica gel chromatography, TLC and finally paper chromatography with Toyo No. 51 paper. Fluorescent bands on TLC plate or paper were scratched or cutted, and extracted with chloroform. After evaporation of chloroform crystals were obtained in a refregerator. Recrystalization was done in ethanol. Yields of the fractions were 26 mg, of F-1, (M.P. 225°) 12 mg, F-2, 2-3 mg, S, respectively.

4) Identification of Ehrlich's positive Substance with Ipomeamarone.

The identity of impomeamarone was carried out by color reactions, paper chromatography, ultra Violet and infra red spectra.

a) Color reaction: The color reaction with Ehrlich's reagent and Liebermann reaction were made with purified ipomeamarone, and the results were shown in Table 1.

Table 1. Color Reactions of Purified Ipomeamarone

Reaction	Result	
	Present	Reported[20]
Ehrlich	pink red	pink red, purple red
Liebmann	purple	purple, purple red

b) Paper chromatography:

Rf values from the various solvents (Methanol, Butanol: Acetic acid: water (45 :5: 50), and butyl acetate) were 0.73, 1.0 and 0.90 respecitively. These values were well agreed with those of reported by Uritani et al (Fig. 12).

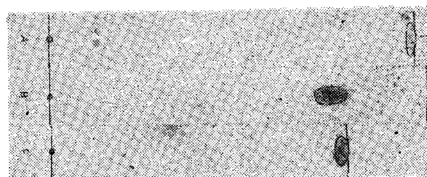


Fig. 12. Paper Chromatography of Purified Ipomeamarone.

A, Butyl acetate
B, Methanol
C, Butanol: Acetic acid: Water (45 : 5 : 50)

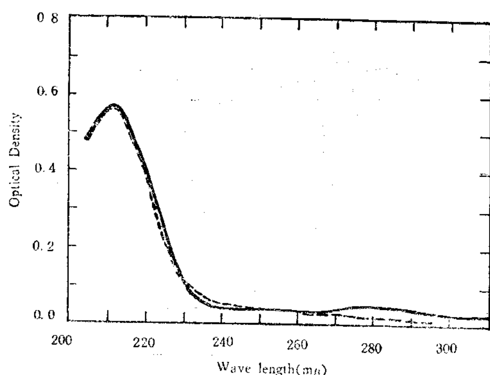


Fig. 13. Ultra Violet Spectra of Ipomeamarone. ——; purified ipomeamarone, ……; authentic ipomeamarone reported.[20]

c) Ultra Violet Spectrum of Purified Ipomeamarone

Purified ipomeamarone was dissolved in 95% ethanol solution and UV spectrum was measured by Beckman DU spectrophotometer as illustrated in figure 13. The spectra was also identical with that of authentic ipomeamarone reported elsewhere.[20]

d) Infra Red Spectrum of Purified Ipomeamarone

Infra red spectrum obtained by KBr plate was silimar to that[30] of reported authentic impomeamarone, in which four characteristic absorption bands in infra-red region at 3.20, 6.40, and 11.42μ, due to its furan structure.

Although slight relative difference in absorption

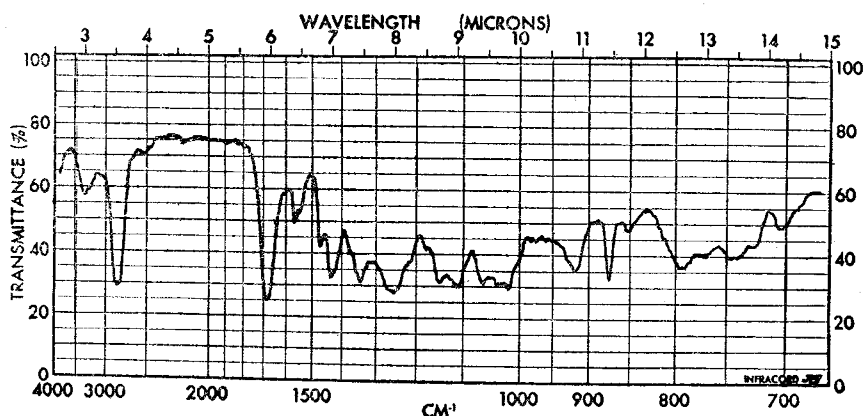


Fig. 14. Infra-Red Spectra of Purified Ipomeamarone.

bands and their sharpness were found, these discrepancies were supposed due to mechanical error possibly caused to zero point adjustment in automatic recorder of IR-spectrophotermeter. And unstable properties of impomeamarone might be responsible for the lack of sharpness in absorption bands. Figure 14 shows the spectrum of present purified ipomeamarone.

5) Identification of Fluorescent Substance F-1 with Umbelliferone.

Paperchromatographic and optical studies of F-1 fraction were almost identical with authentic umbelliferone as shown in table 2, through figure 15 and 16.

This series of results shows clearly that identification of the isolates from soft-rotted sweetpotato

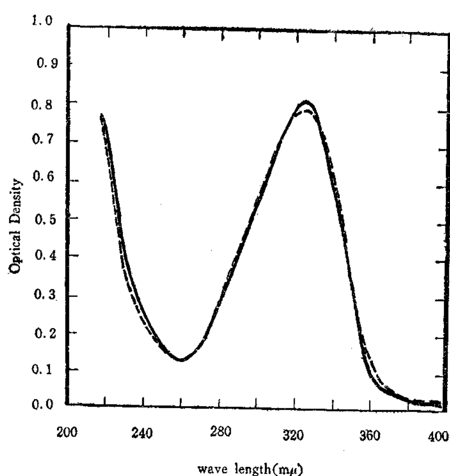


Fig. 15. Ultra violet absorption spectra of FM (——) and Umbelliferone(……).

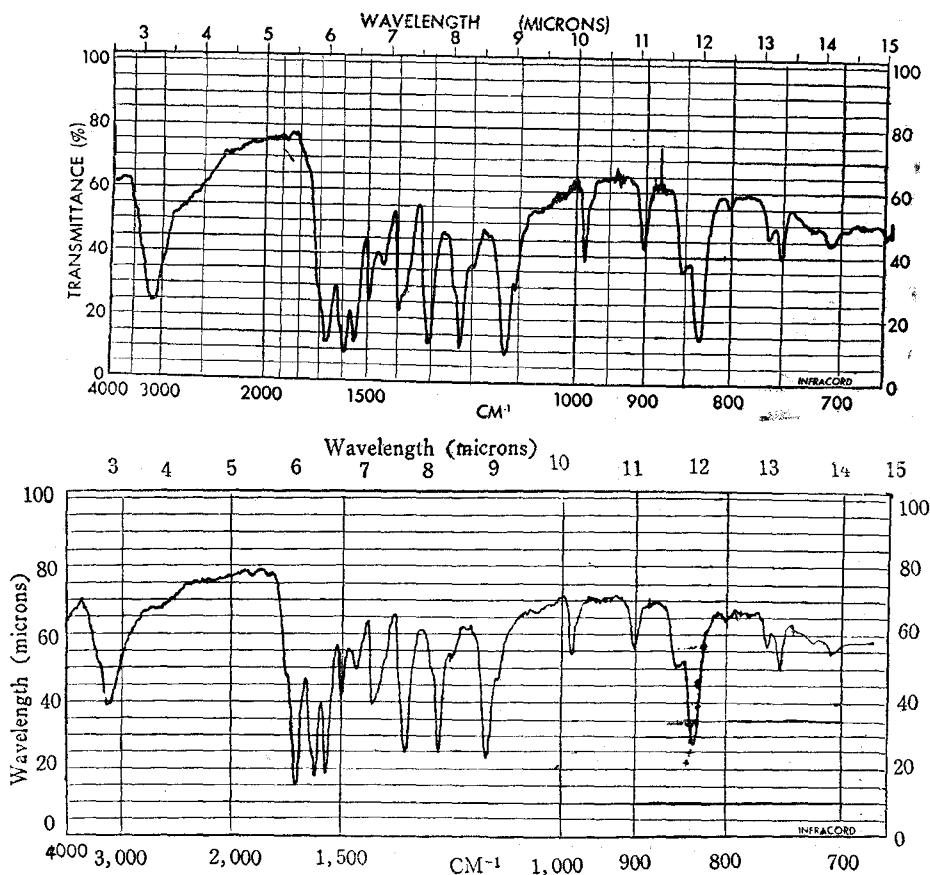


Fig. 16. Infrared Spectra of F-1 (a) and Authentic Umbelliferone (b).

is possibly to be concluded; Ehrlich's positive substance as ipomeamarone and fluorescent compound F-1 as umbelliferone. The latter was further justified by detection of methoxyl group, or F-1 showed a positive reaction with resulting light orange color due to the formation of mercuric iodide in the *Zeisel's* Alkoxyl method[34].

Table 2. Rf values of Fluorescent Compound

Solvents	Rf values	
	F-1	Umbelliferone
Ethanol : NH_4OH:H_2O (80 : 5 : 15)	0.53	0.54
Butanol : Acetic Acid : Water (4 : 1 : 1)	0.93	0.94
Water sat urated but anol	0.90	0.92
5% Acetic acid	0.405	0.405

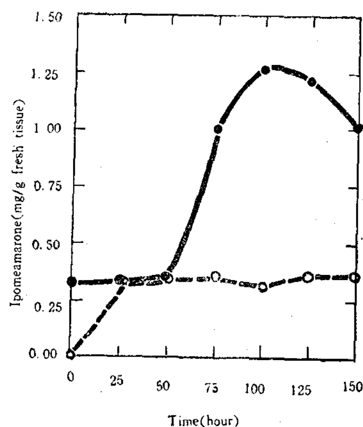


Fig. 17. Time course synthesis of Ipomeamarone ●—●; incubated with *R. nigricans*, ○--○ incubated without organism.

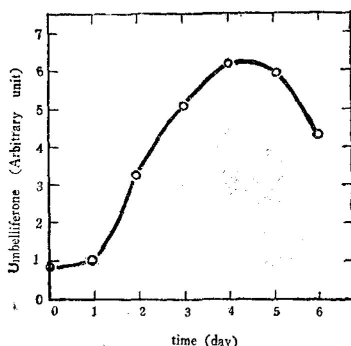


Fig. 18. Time Course Synthesis of Umbelli ferone.

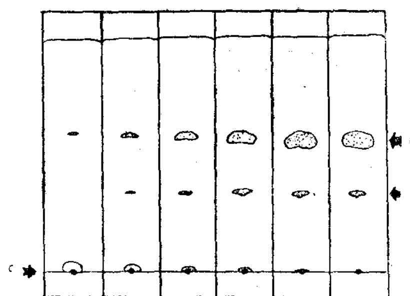


Fig. 19. TLC showing time course biosynthesis of fluorescent compound. a, b and cindicate Umbelliferone, unidentified fluorescent compound and chlorogenic acid, respectively.

6) Time Course Biosynthesis of Ipomeamarone and Umbelliferone.

It was interested to know whether ipomeamarone and umbelliferone were synthesised pararelly with the softening of tuber.

Sweet potato slices were inoculated with *Rhizopus nigricans* and incubated at 25°C for different period of time and subjected ipomeamarone and umbelli ferone analysis.

At the begining stage of infection, no ipomeamarone detected on TLC plate. The syntheis started after 24 hours inoculation and continued until the fourth day. Thereafter the biosynthesis began to level off.

The analysis was made every 24 hours interval for one week as shown in figure 17, and biosynthesis of umbelliferone was also analyzed spectrophotometrically at 320 mμ as shown in figure 18. Umbelliferone was began to synthesized at the 24 hours inoculation until maximum synthesis was attained at the fifth day and thereafter the synthesis was decreased like that of ipomeamarone.

The other mino spotr on TLC is also believed to be synthesized similary with that of umbelliferone, although its identity was not investigated. Qualitative estimation was possible on the TLC plate as shown in figure 19.

7) Ipomeamarone as Enzyme Inhibitor

Sweetpotate is a good raw material in alcohol fermentation. When soft-rotted tuber was subjected to an enzymatic hydrolysis, less alcohol yield by yeast was attained.[38] This was supposed to be due to an inhibitory action of some component in sweet potato to enzyme concerned. alcohol fermentation. α-amylase, convenience, was examined for the with alcohol extract from soft-rotted sweetpotato and the enzyme was inhibited appreciably. This inhibitory reaction was resulted, from the xameination both with ether soluble fraction and insoluble fluorescent substances. Figure 21 shows the inhibitory degree of the ether soluble Ehrlich's positive substances, which include ipomeamarone as one of the main responsible spot on TLC strip.

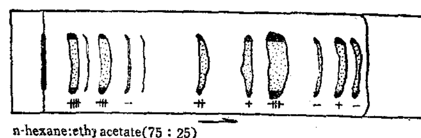


Fig. 20. Chromatostrip showing the Degree of Enzyme Inhibition; + denotes the degree of the inhibition, —represents no inhibition. Shaded parts were sprayed by Ehrlich's reagent to know the locations of compound 5on TLC strip.

Purified ipomeamarone was studied to elucidate the inhibition kinetics, or inhibition type, using α-amylase as a model enzyme. Effect of ipomeamarone in enzyme activity was apparently demonstrated as illustrated in figure 20.

On the other hand inhibition type was determined by ploting 1/V versus 1/S, and 1/V vs. i. Figure 22 and 23 gives the results, which have been ploted

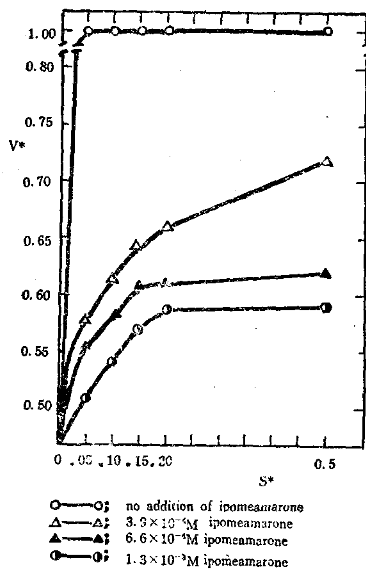


Fig. 21. Effect of Ipomeamarone to Enzymatic Hydrolysis of Starch by α-amylase.

* 1, V; reaction velocity expressed by DB(40°, 30′) (Blue value[36])

* 2, S; substrate concentration(%)

in graphical form. In figure 23, double reciprocal plots of 1/V vs. 1/S, the slop of the curve remains constant, but the intercept was altered. These parallel curves represent the characteristics of uncompetitive inhibition. Another kinetic diagonistic for uncompetitive inhibition is that the double reciprocal plot 1/V vs. i, the intercept was also varied with inhibitor concentration (Fig. 23).

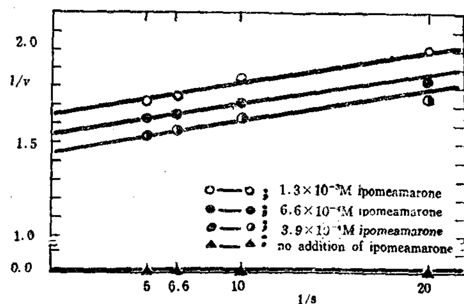


Fig. 22. Graphical plot showing inhibition type; 1/V vs. 1/S.

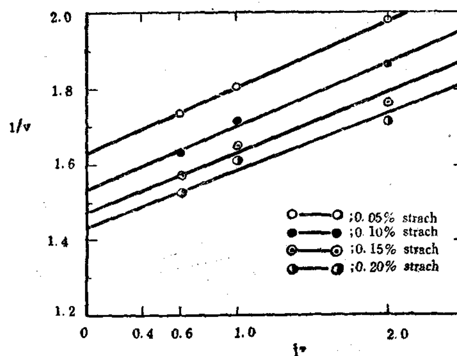


Fig. 23. Graphical plot of 1/V vs. i.

* i; inhibitor concentration (ipomeamarone mg/ml)

8) Effect of Ipomeamarone on the Oxygen Uptake by Yeast.

As shown in figure 24, addition of ipomeamarone in the medium of yeast, oxygen uptake was noticeably accelerated. This acceleration effect was linearly proportioned to the concentration of ipomeamarone. This effect was compared with that of DNP, an uncoupler, in other word the addition of

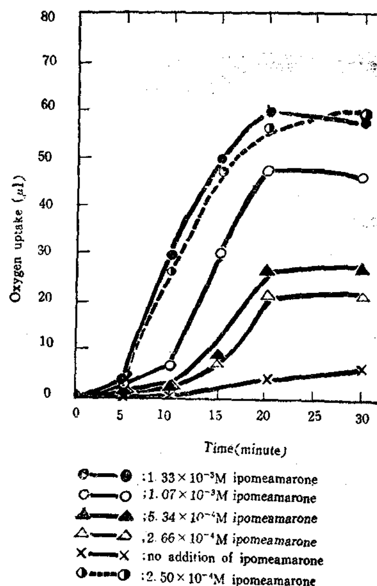


Fig. 24. Effect of Ipomeamarone to Oxygen Uptake by Yeast.

ipomeamarone showed the same effect of the uncoupling reagent.

IV. DISCUSSION

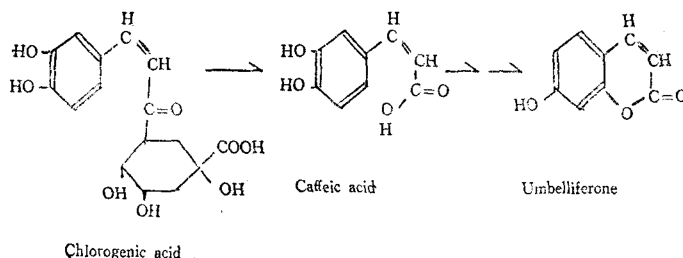
Ipomeamarone and umbelliferone were isolated from soft-rotted sweetpotato. Several Ehrlich's positive substances and another minor fluorescent substance were detected. These findings attract several biochemical attentions to soft decayed sweetpetato tuber. Previously, it had been thought only a strong pectolytic enzyme responsible for the rapid softening phenomena in tubers, followed by the penetration of mycel of fungus. Identification of these abnormal metabolites in soft-rotted tuber strongly suggests that an appreciable biochemical change could accompany the developement of soft-rot in the tuber. Although all the metabolites produced have not been clearly studied for their identity, the many Ehrlich's positive substances from the decay indicate that a biosynthetc pathway of black rotted sweetpotato similar to that proposed by Uritani et. al.,[26] could arise in the soft decay.

In addition to ipomeamarone, ipomeamanine, furan-β-carboxylic acid, batatic acid and relative substances were conceivably produced by the pathway involving mevalonic acid mediated by acetoacetyl CoA and further oxidation leading to batatic acid, ipomeamarone and ipomeamanine (Fig. 1).

Observation of the time course production of ipomeamarone supports the view that the same or at least a similar consideration, as in the case of black-rotted tuber, appears to be accepted in soft-rotted sweetpotato.

The biosynthetic pathway for the formation of coumarins, formulated by Rudkins, however,[28] should be discussed before applying it to softr-otted tubers. Uritani[39] understood that the same metabolic pathway in the healthy plant was generally accepted without any modification to correspond to the one in the plant, including black-rotted sweet potato. This was proved to be true by isolating metabolites of the pathway from the diseased tuber; umbelliferone as coumarin compound, chlorogenic acid as polyphenol. Thep present observation of soft-rot, however, showed no accumulation of chlorogenic acid and rather decreased their content which had been present in the healthy tuber[43] as illustrated in figure 22. This discovery necessitated a discussion of the proposed pathway to the following points. That no chlorogenic acid accumulated during the decay is assumed to be due to an alteration of the pathway by a blocking of the metabolic step involving the conversion of cinnamic acid either to cinnamoylquinic acid or to p-coumaric acid which leads to chlorogenic acid, as depicted in Fig. 2. An alternative consideration could be made wiht a postulation of the possibile conversion of chlorogenic acid to umbelliferone or to coumarins. Although such a biosynthetical conversion has not been reported nor has any illustration of the transferation of the hydroxyl group in the plant aromatic ring been available, the following conversion of chlorogenic acid to umbelliferone is thought to be cenceivable and will open for further investigation.

An additional possibility explaining the biochemical discrepancy of the accumulation of chlorogenic acid could be that the polyphenol is oxidized to caffeic acid, follewed, probably in the course of experimental procedure, by a polymerization to a new polyphenol compounds, such as lignin, whichis dark brown in color. This color change was also



noticed during the experimental procedure.

Abnormal metabolites have been studied in their biochemical and other properties: ipomeamarone was reported as a toxic substance to animals and humans[40] and was known to show a effect to the saccharification of starch in the aceton-butanol fermentation,[41] and an uncoupling action.[42] The sweetpotato has been one of the important raw material in the industrial fields for use in the of manufacturing, alcohol and glucose. Great quantities of sweetpotatoes were inevitable to subject the fungal casualties, causing a serious decrease in the industrial yield. The present work also aimed to elucidae the causes of unfavorable effects, due to softrot decay.

In order to elucidate the unfavorable effects of the soft-rotted sweetpotato, all the isolates obtained from the tuber were tested with α-amylase as a model enzyme; the results indicated that Ehrlich's positive substances were responsible for the inhibitory action. Ipomeamarone and another substance, which was not identified, showed strong inhibitory action on the hydrolysis of starch by the enzyme. The inhibitory type was understood to be an uncompetitive one, as shown in figure 22.

In relation to the uncoupling action with yeast cell, ipomeamarone was assumed to selectively inhibit enzymes concerned with oxidative posphorylation in the cell; the investigation of this possibility must be the subject of future research.

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要 約

Rhizopus nigricans 에 感染된 고구마의 生化學的 性質을 究明하기 위하여 軟腐고구마로부터 異常代謝產物을 分離하고 그 性質에 關하여 實驗한 結果를 다음과 같이 要約한다.

1. 軟腐고구마로부터 健全한 고구마에는 없는 異常代謝產物로서 19 個의 Ehrlich's 試藥에 對한 陽性反應物質과 3 個의 螢光性物質의 存在를 薄層크로마토그라프로 確認하였다.

2. 異常代謝物質中에서 그 生成量이 가장 많은 物質로서 Ipomeamarone 과 Umbelliferone 을 分離하고 이들의 精製와 同定을 行하였다.

3. Ipomeamarone 과 Umbelliferone 의 經時的 生成量을 觀察하고 이들의 可能한 生合成 經路에 關하여 考察하였다.

4. 軟腐고구마의 異常代謝物中 Ipomeamarone 과 이의 類似物質은 α-amylase 에 對한 活性을 抵害하였으며 그 抵害型은 非競爭的이었다.

5. 軟腐고구마로부터 분리한 Ipomeamarone 은 Yeast 의 呼吸을 促進시켰으며 酸化的 燐酸化反應의 聯合解除劑인 2, 4-dinitrophenol 과 同一한 効果를 나타내었다.

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