

# 貯蔵ピーマン果実の低温障害に関する生理化学的研究(第4報)

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## Physiological and Chemical Studies of Chilling Injury in Pepper Fruits

### IV. The Identification and the Changes of Content of Lignin Aldehydes in Pepper Peels and Seeds Stored at 6°C and 20°C

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#### Summary

1. The present experiments show the identification and the changes of content of lignin, which is considered to be closely related to the hardness of plant tissue in pepper fruits. And the relation to the accumulation of phenolic substances in pepper seeds by low temperature storage reported in previous paper is discussed.

2. The lignin in the peels and seeds of pepper fruits was isolated as lignin aldehydes by the method of alkaline nitrobenzene oxidation described by Stone and Blundell. The quantitative determination of the aldehydes was carried out using paper chromatography.

3. The lignin aldehydes were detected by spraying on paper with 2% 2,4-dinitrophenylhydrazine in 2N HCl. Three spots were found as lignin aldehydes of peels and seeds. One spot was identified as vanillin by the determination of maximum absorption, Rf value, and color reaction of hydrazone while the other spots were unidentified substances.

4. The vanillin content of pepper peels was fairly lower than that of seeds. At 6°C, the vanillin content of peels decreased by half of initial value 2 days after and then increased rapidly during subsequent storage period. The content in 20°C-stored fruits, on the contrary, increased gradually during 2 days of storage. The vanillin content in pepper seeds showed 685  $\mu\text{g/g}$  fresh weight before storage, and in the storage at 6°C it decreased rapidly to 417  $\mu\text{g/g}$  fresh weight after 2 days, and then increased to reach to a maximum of 807  $\mu\text{g/g}$  fresh weight after 7 days and the high level followed constantly during storage periods, while the content of that stored at 20°C increased gradually and reached a maximum after 7 days.

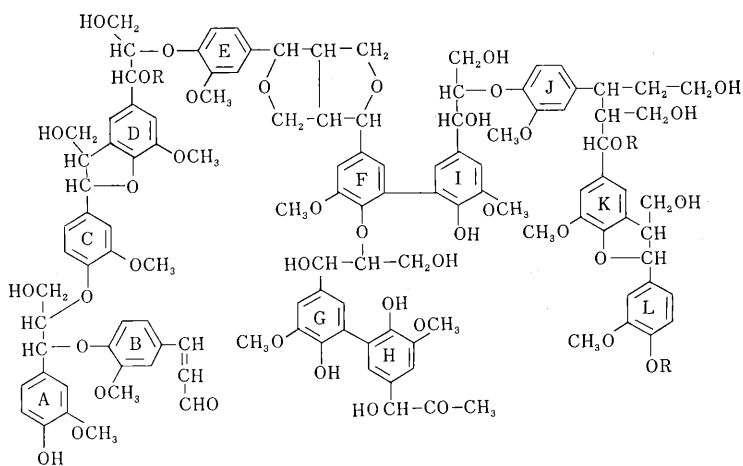
#### Introduction

Lignin is very complex substance and have not been well understood till present. Lignin is supposed to have structure as shown below.

Lignin is a polymer which contains a large number of aromatic nuclei and its molecular weight is considered to be above fifty thousands, and that phenylpropane as lignin base is not only one kind, but it is different among plant groups.

Lignin of needle-plant is

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Structure elements of guaiacyl lignin.

(From Bonner and Varner, 1965.)

formed from guaiacylpropane, and one of wide-leaf plant is formed from guaiacylpropane and syringpropane, and in rice plant it has been known to be formed from p-hydroxyphenylpropane, syringpropane and guaiacylpropane.

The study concerning biosynthesis of lignin was reported by Freudenberg(2). After that, it has been developed more rapidly by using tracer techniques of  $^{14}\text{C}$ -labeled substance. Brown *et al.*(1) has reported that shikimic acid- $^{14}\text{C}$  was incorporated rapidly in lignin. Further, they postulated that  $^{14}\text{C}$ -labeled phenylalanine and tyrosine were incorporated in lignin of wheat after 24 hours of incubation. From the results of this experiment, they has assumed that phenyl-ring of aromatic amino acid would change into that of lignin.

Koukol *et al.*(4) found an enzyme of phenylalanine ammonia-lyase (PAL) in sweet clover that catalyzes the formation of trans-cinnamic acid from phenylalanine, and subsequently Neish(8) recognized tyrosine ammonia-lyase(TAL) that converts tyrosine to paracoumaric acid in Sorgham seedling. From this fact, it has been confirmed that lignin is metabolized through the shikimic acid pathway described by Brown *et al.*(1).

In this report changes of lignin, which is considered to be closely related to the hardness of plant tissue, were determined during storage of pepper fruits and the relation to the accumulation of phenolic substances in pepper seeds by low temperature storage, reported in previous paper, is discussed.

### Materials and Methods

Pepper fruits (Hyōgo-midori) were obtained from Tarumi, Hyōgo prefecture on October 9, 1969, and stored at 6°C and 20°C under darkness.

#### *Analysis of lignin aldehydes.*

Five grams of pepper seeds and peels were ground with a Waring Blender in 75% ethanol and thoroughly extracted with 75% ethanol under reflux on

the steam bath. The combined filtered extracts were evaporated in a rotary evaporator. The residue obtained was extracted with 5 ml of ethanol and used for determination of phenolic acid.

Alkaline nitrobenzene oxidation was carried out using the method of Stone and Blundell (10). To 50 mg of dried ethanol insoluble residue 0.1 ml of nitrobenzene and 2 ml of 2N NaOH were added in a stainless steel bomb. After the mixture was heated at 160°C for 2 hr in oil bath, the bomb was cooled in a streaming water and its contents were centrifuged with 15000 rpm for 10 min in order to separate the cell debris. Two ml of supernatant was transferred to the test tube and repeatedly extracted by shaking with 400~500 ml of ether. The extracts were discarded and aqueous layer was acidified with 2 ml of 2N HCl and repeatedly extracted with 100 ml of ether. This ether extract contained the lignin aldehydes resulting from oxidation of lignin.

*Quantitative determination of lignin aldehydes.* The quantitative determination of lignin aldehydes was carried out on paper of 40 cm wide and 40 cm long (Tōyō filter paper No. 51 A). A line was drawn across the paper at 5 cm from one end. 0.02 ml of the mixture

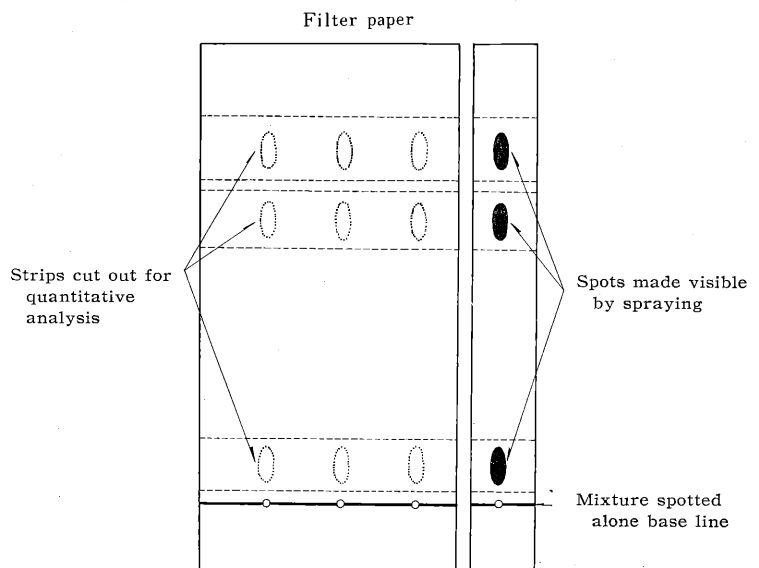


Fig. 1. Method for cutting chromatogram for quantitative analysis.

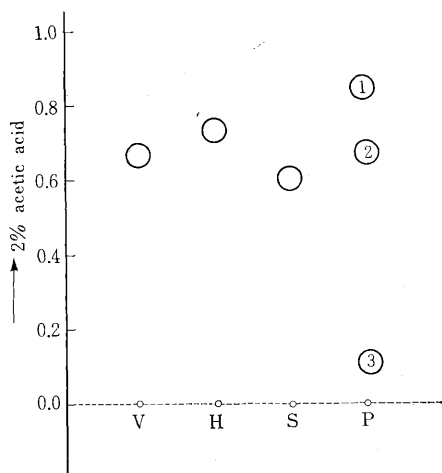


Fig. 2. Separation of vanillin(V), p-hydroxybenzaldehyde(H), syringaldehyde(S) and the production by alkaline nitrobenzene oxidation of pepper seeds(P).

to be analyzed was spotted along the line. After developing with 2% acetic acid for 3 or 4 hr., the lignin aldehydes were detected by spraying the paper with a solution of 2% 2,4-dinitrophenylhydrazine in 2N HCl. A blank was also taken (Fig. 1). The compounds in the area containing vanillin, syringaldehyde and p-hydroxybenzaldehyde were eluted from the paper chromatograms with 5 ml of ethanol containing 0.2% KOH solutions. Optical densities were measured at 354  $m\mu$  for vanillin, 336  $m\mu$  for p-hydroxybenzaldehyde and 370  $m\mu$  for syringaldehyde. UV absorption spectrum was compared with those from the spectrum of authentic aldehydes.

## Results

### *Chromatographic properties and spectral characteristics of authentic vanillin, p-hydroxybenzaldehyde and syringaldehyde*

Fig. 2 shows the paper chromatogram of authentic lignin aldehydes (V=vanillin, P=p-hydroxybenzaldehyde and S=syringaldehyde) and products of alkaline nitrobenzene oxidation of pepper peels and seeds which were detected by spraying with 2% 2,4-dinitrophenylhydrazine solution.

Table 1 shows the result of Rf value, maximum absorption and color reaction of hydrazone of authentic vanillin, p-hydroxybenzaldehyde and syringaldehyde. Rf value

Table 1. The characteristic reaction of vanillin, p-hydroxybenzaldehyde and syringaldehyde.

Materials	Rf value	Maximum absorption	2,4-dinitrophenylhydrazine reaction
Vanillin	0.66	354	brown
P-hydroxybenzaldehyde	0.71	336	brown
Syringaldehyde	0.60	370	brown

Table 2. The characteristic reaction of the product by alkaline nitrobenzene oxidation of pepper seeds.

Spot No.	Materials	Rf value	Maximum absorption	2,4-dinitrophenylhydrazine reaction
1	unidentified substance	0.83	285	brown
2	vanillin	0.64	354	brown
3	unidentified substance	0.13	271	brown

and maximum absorption of authentic vanillin were presented as 0.66 and 354  $m\mu$ , those of p-hydroxybenzaldehyde were 0.71 and 336  $m\mu$ , and those of syringaldehyde were 0.60 and 370  $m\mu$ , respectively.

### *Characteristic reaction of the products by alkaline nitrobenzene oxidation of pepper seeds*

As shown in Fig. 2, three spots were detected by spraying on paper with 2,4-dinitrophenylhydrazine solution. Spot 1 was unidentified substance, its Rf value was 0.83 and maximum absorption was 285  $m\mu$ . Spot 2 was 0.64 and 354  $m\mu$  corresponding with authentic vanillin, Spot 3 was unidentified substances. Its value was 0.13 and maximum absorption was 271  $m\mu$ .

Fig. 3 shows the absorption spectra of authentic vanillin and the compound eluted with 5 ml ethanol containing 0.2% KOH from the area containing spot 2.

The absorption spectrum of spot 2 coincided with that of authentic vanillin, and the flavor of spot 2 possessed characteristics of vanillin.

From the result mentioned above, spot 2 was identified as vanillin, while spot 1 and 3 were unidentified substances.

### *The vanillin content of pepper peels stored at 6°C and 20°C*

The quantity of each lignin aldehyde of

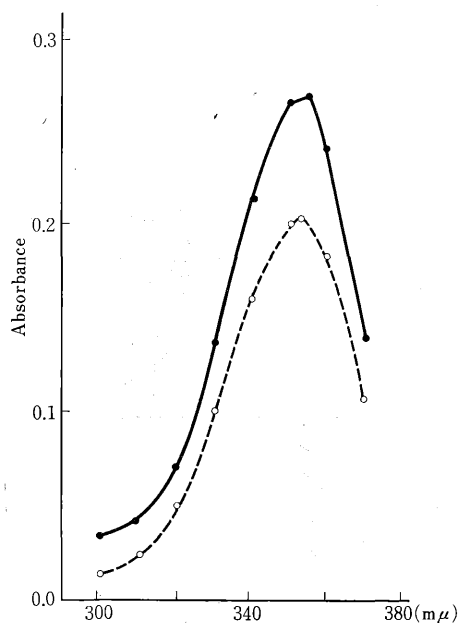


Fig. 3. Absorption spectra of vanillin (○---○) and the product by alkaline nitrobenzene oxidation of pepper seeds (●—●).

Table 3. The changes of vanillin content produced by alkaline nitrobenzene oxidation of pepper peels stored at 6°C and 20°C.

Temperature	Days in storage			
	0	2	7	14
6°C	76*	39	83	102
20°C		67	93	95

\*  $\mu\text{g/g}$  fresh weight.

Table 4. The changes of vanillin content produced by alkaline nitrobenzene oxidation of pepper seeds stored at 6°C and 20°C.

Temperature	Days in storage					
	0	2	4	7	14	21
6°C	685*	417	802	821	645	714
20°C		737	749	807	670	761

\*  $\mu\text{g/g}$  fresh weight.

pepper peels and seeds was determined with the method shown in Fig. 1.

The result of the determination of vanillin in pepper peels is shown in Table 3. The

vanillin content at initial of storage was 76  $\mu\text{g}$  per g fresh weight and in cold storage the content decreased rapidly and became 39  $\mu\text{g}$  per g fresh weight after 2 days reaching a minimum, then it turned to increase sharply showing 102  $\mu\text{g}$  per g fresh weight after 14 days. The content in 20°C-stored fruits showed slight decrease at first, then increased gradually during subsequent storage, but after 14 days the vanillin content in 20°C-stored fruits was a little lower than that in 6°C-stored fruits.

*The vanillin content of pepper seeds stored at 6°C and 20°C*

Table 4 shows the vanillin content of pepper seeds. The vanillin content in pepper seeds showed 685  $\mu\text{g}$  per g fresh weight before storage, and in storage at 6°C it decreased rapidly to 417  $\mu\text{g}$  per g fresh weight after 2 days, and then increased to reach to a maximum of 807  $\mu\text{g}$  per g fresh weight after 7 days, and the high level followed constantly during storage periods, while the content of that stored at 20°C did not show any marked decrease throughout storage period.

### Discussion

In our previous paper(5), it have been reported that pepper fruits suffered physiological injury at 0~10°C and the symptom of typical surface pitting was observed, and that the peels became hard during low temperature storage. In those stored at 1°C, the browning of seeds appeared after 2 days and the longer pepper fruits were stored at low temperature, the more degree of seeds browning increased.

In general, it is known that lignin is a major constituent of wood, accounting for 22~34% of most woods, the remainder being cellulose, hemicellulose, and extractives, and that it is considered to be related in hardness of fruits and vegetables(3). Brown *et al.*(1) has reported that the biosynthesis of lignin is formed through shikimic acid pathway. In our previous paper(6), the main phenolic substances of pepper seeds were identified as chlorogenic acid which is formed through shikimic acid pathway. The chlorogenic acid was accumulated rapidly at low temperature storage, and the changes of both the shikimic acid and tyrosine which are considered

as precursor of chlorogenic acid coincided with the accumulation of chlorogenic acid. On the other hand, phenylalanine ammonia-lyase (PAL) known as the key enzyme of biosynthesis in phenylpropanoids such as chlorogenic acid, caffeic acid and lignin was activated sharply at low temperature storage. From above result, we have suggested that PAL activity would be closely related with the accumulation of chlorogenic acid in pepper fruits.

As shown in Fig. 2, three spots were detected on the paper chromatograms. Two among three spots were unidentified substance. One spot was identified as vanillin (Table 2 and Fig. 3). The vanillin content of pepper peels was fairly lower than that of seeds (Tables 3 and 4). At low temperature storage (6°C), the vanillin content of peels decreased by half of initial value 2 days after and then increased rapidly during subsequent storage period. The content in 20°C-stored fruits increased gradually during 2 days of storage, but did not decrease so sharply as that of cold storage.

The vanillin content of pepper seeds showed the same tendency as that of peels. At low temperature storage, the vanillin content decreased rapidly 2 days after, and then increased gradually. Consequently, the vanillin content of pepper peels and seeds was supposed to decrease rapidly 2 days after.

As shown in previous paper(5,6,7), the pepper seeds turned brown after 2 days of cold storage and chlorogenic acid content increased at the same time, further, phenylalanine ammonia-lyase was activated after 2 days of storage. We have an interest in the fact that vanillin decreased after 2 days of cold storage, that is, the content of vanillin and phenolic substances has exactly reverse relation. From this result, we have suggested that the metabolism of lignin and phenolic substances may trend to produce of phenolic substances at the early days of storage. Then the production of lignin is repressed, and the phenolic substances is accumulated. Once the seeds browning is observed clearly, it may be that the metabolism trend to produce of lignin.

Now, lignin contained in plants has been thought as end product, and it is said that

the transfer of lignin in plant does not occur at all, but from the results of our study, the transfer of lignin was assumed. Ogawa *et al.* (9) has reported that the degree of dyeing of potato tissue slices by the method of histochemical dyeing is very different during storage. This data suggested that lignin may be transfer. The problem about the transfer of lignin should investigate further.

As the factor concerning biosynthesis of lignin, it is considered that peroxidase is closely connected with lignin biosynthesis, and that the peroxidase is even important enzyme concerning browning of seeds as well as polyphenol oxidase. We must study further about this enzyme.

The possibility of existence of lignin transfer system was supported by tracer studies, *i.e.*, administered phenylalanine-U-<sup>14</sup>C incorporated rapidly into vanillin in pepper fruits and then marked decrease of active lignin was observed during the incubation. Detail will be reported.

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## 貯蔵ピーマン果実の低温障害に関する生理化学的研究 (第4報)

ピーマン果皮および種子のリグニンアルデヒドの検索と貯蔵中の変動

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### 摘 要

ピーマン果実を 0~6°C に貯蔵すると短時日で種子のかつ変が生ずる。また果皮面にも pitting が生じ、果色も黒ずみ、貯蔵が長期におよぶと果皮が硬化する。本研究はこのような低温障害と関連し phenylalanine ammonia-lyase や tyrosine ammonia-lyase の活性およびかつ変基質物質と密接に関係するリグニンについて調べ、かつ変基質物質の生成とリグニン生成との関係を考察したものである。

1. 果皮および種子のリグニンアルデヒドを検索したところ、3点検出され、1点はバニリンと同定したが、他の2点は同定できなかつた。

2. 果皮のバニリン含量は種子よりもはるかに少なく、6°C—2 日後に種子と同様低下したが、その後急増

した。20°C 区では2日以後漸次増加の傾向を示した。

3. 種子のバニリン含量は 6°C—2 日後に当初の約 1/2 に減少し、以後急増し、貯蔵後半までかなり高い値を保持したのに対し、20°C 区では 6°C 区でみられた貯蔵2日後の減少はみられず、全貯蔵期間を通じてかなり高い値を保持した。

4. 以上の結果から、バニリンが果皮および種子の主要リグニンアルデヒドであること、およびその含量が 6°C—2 日後に当初の約 1/2 に低下するのにかかわらず、種子かつ変基質が増加する時期であることから、リグニンの生成とかつ変基質物質の生成の間には相反する関係のあることがわかつた。