Inhibition of ACC-dependent Ethylene Production in Apple Fruit Discs by a Cell Wall Component from Peach Fruit

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Summary

Substance(s) inhibitory to ACC-dependent ethylene production in the alcohol-insoluble solids (AIS) fraction from peach fruit was assessed in apple fruit discs.

The supernatant from AIS digested with pectolyase Y-23 in distilled water inhibited ethylene production in apple fruit discs. Separation of the supernatant on a PD-10 column indicated that the inhibitory substance(s) was associated with the low molecular weight fraction. The inhibitory effect of the supernatant was lost upon dialysis against distilled water. The inhibitory fraction appeared as a single entity ($R_f=0.44$) when separated by thin layer chromatography. Inhibitory substance(s) was also released by macerolase and cellulase digestion or by autocatalytic digestion of freshly prepared peach fruit homogenate. These data indicate that peach fruit may release a substance(s) which inhibits ethylene production.

Introduction

Fruit ripening is a complicated phenomenon involving many coordinated steps. Transgenic tomato fruit having the antisense gene of pTOM 13 [later verified as 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase] revealed a delay of the ripening process accompanying the inhibition of ethylene production (Hamilton et al., 1990). Thus, ethylene has been thought as a trigger of fruit ripening. Applications of aminoethoxyvinylglycine (2-amino-4 [2-aminoethoxy]-*trans*-3-butenoic acid) (AVG) has been examined for the delay of ripening and enhancement of fruit quality (Bangerth, 1978). This benefit is derived from the inhibition of ethylene production by AVG via its suppression of the conversion of *S*-adenosylmethionine (SAM) to ACC (Yang and Hoffman, 1984).

Ripening (softening) of peach fruit advances dramatically resulting in rapid deterioration of fruit quality. In the process the cell walls are rapidly broken down, most particularly into polyuronide components (Pressey et al., 1971). It has been suggested that fragments derived from plant cell walls can serve as regulatory molecules including regulation of ethylene production (Albersheim et al., 1983). It has also been suggested that cell wall fragments derived from peach fruit may have some functional role in the ripening (softening) process and regulation of ethylene production. Tong et al. (1986) reported that cell wall fragments of pear cell suspension cultures digested with macerolase led to a rapid and transient production of ethylene by the cells. Similarly, pectic materials stimulated ethylene production when vacuum-infiltrated into whole mature green tomato fruit (Tong and Gross, 1990). Pectic oligomers also induce and regulate ethylene biosynthesis in cultured pear cells (Campbell and Labavitch, 1991a) and tomato pericarp discs (Campbell and Labavitch, 1991b).

In this paper, we report the inhibitory effect on ethylene production by a fraction derived from alcohol-insoluble solids (AIS) of peach fruit, and partial purification of this fraction.

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Materials and Methods

Chemical

AVG and ACC were obtained from Sigma Chemical Co., St. Louis, USA ; pectolyase Y-23 from Seishin Pharmaceutical Co., Ltd., Tokyo, Japan ; and glucoamylase from Seikagaku Corp., Tokyo, Japan.

Preparation of AIS

Peach ('Hakuho' and 'Hakuto') fruits for the extraction of AIS were harvested from mature trees growing at the Chiyoda Experimental Orchard of the Fruit Tree Research Station (Tsukuba). The fresh pulp tissue was cut into small pieces and homogenized in 3 volumes of 80% ethanol. After pH adjustment to about 7 with 0.1 N NaOH, the homogenate was heated at 80 °C for 10 min, and then centrifuged at 10,000×g for 15 min. The pellet was washed twice with 2 volumes of 80% ethanol and the residue was dried under vacuum.

Partial purification

AIS (equivalent to 40 g fresh weight) was resuspended in 30 ml of distilled water and digested with 5 mg glucoamylase at 37 °C for 3 hr. The reaction was stopped by boiling the mixture for 15 min. The mixture was then passed through a glass filter, and the residue was resuspended in 25 ml distilled water and incubated with 20 mg pectolyase Y-23 at 37 °C for 24 hr. The mixture was then boiled for 15 min, and passed through a glass filter. The filtrate fraction was collected and concentrated to 2.5 ml by vapour mix. The concentrated filtrate was passed through a PD-10 column (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) as specified in the manufacturer's manual and the low molecular weight fraction (6~12 ml) was collected. This fraction was further concentrated into a small volume by vapour mix and developed on thin layer chromatography (TLC) (Kieselgel 60 F₂₅₄, E. Merck, Frankfurter Strasse 250, Germany). The solvent for the 1st development step was *n*-butyl alcohol : pyridine : distilled water = (6 : 4 : 3). The same solvents at 1 : 1 : 1 were used in the 2nd and 3rd development steps. In a preliminary experiment, we found that the inhibitory effect on ethylene production was in the low R_f values. Subsequently, fractions of average R_f = 0.24 (# 1) and 0.44 (# 2) under UV light

(A₂₅₄) were recovered from TLC plates, using the same 1 : 1 : 1 solvent mentioned above. The recovered fractions were evaporated completely under vacuum and tested in the ethylene assay.

Autocatalytic experiment

Fresh peach fruits (300 g) were homogenized in distilled water (600 ml) and centrifuged at 750×g for 20 min. The pellet fraction was resuspended in 40 ml of distilled water. Ten ml of this mixture were added into AIS with 3 drops of toluene (as an antiseptic) and incubated for 20 hr at 30 °C. After incubation, the mixture was boiled and passed through a glass filter. The filtrate was tested in the ethylene assay. Boiled peach fruit homogenates were used as controls.

Ethylene assay

The ethylene assay solution consisted of 3.5 ml of 0.1 M Hepes-KOH buffer (pH 5.9), 1.5 ml of inhibitor solution or 1.5 ml of distilled water (as control), 20 µl of ACC (stock solution ; 1 mg·ml⁻¹), 100 µl of AVG (stock solution ; 1 mg·ml⁻¹), mannitol for osmoregulation (final concentration 0.6 M) and about 1 g apple fruit discs (2 pieces of ø 0.85 cm×1 cm) in a glass tube (ø 1.5 cm×10.5 cm). Apple ('Fuji') was used for the assay because of ease of handling and availability throughout the year. After mixing well, the assay tubes were sealed tightly with a silicone cap, and left in the dark at room temperature. Ethylene production rates were determined at 2-hour intervals. A 3 ml gas sample was withdrawn from the gas phase using a syringe fitted with a hypodermic needle, and analyzed at 80 °C in a gas chromatograph equipped with an alumina column and FID and quantified by comparison with the retention time and peak area of standard samples. Immediately after sampling the test tubes were opened to allow in fresh air and sealed again for the next incubation period. As ethylene production in the control varied due to the physiological differences of apple fruit discs employed in the assay, ethylene production was determined in at least 2 replications and results are generally presented as % of the control value.

Results and Discussion

Pectolyase Y-23 digest of AIS from unripened (over 8 lb·in⁻² firmness by penetrometer fitted

with an 0.8 cm probe) and ripened (almost 0 lb·in⁻²) peach fruit strongly inhibited ethylene production by apple fruit discs (Fig. 1). Macerozyme and cellulase digests also effectively inhibited ethylene production (data not shown). After 8 hr incubation, macerozyme and cellulase digests inhibited ethylene production by 32 and 29%, respectively relative to the control. After partial purification of the pectolyase digest on a PD-10 column, we found that the low molecular weight fraction (6~12 ml) was the active component in inhibiting ethylene production (Table 1). Dialysis against distilled water of the supernatant from pectolyase Y-23 digestion caused complete loss of its inhibitory effect (Table 2). Accordingly, the molecular weight of the inhibitory substance(s)

Fig. 1. Effect of the AIS extracts after pectolyase Y-23 digestion of peach fruit on ethylene production by apple discs.
A ; control. B ; unripened peach fruit. C ; ripened peach fruit.

was estimated to be less than 3,000. Recovery of the separate fractions [Rf = 0.24 (#1) and Rf = 0.44 (#2)] from TLC plates indicated that fraction in #2 had the highest inhibitory effect (Fig. 2). Spectrophotometric scanning of fraction (#2) from A₇₀₀ to A₁₉₀ indicated a small absorbance peak at about A₂₅₀ suggesting the existence of phenolics (data not shown). The results from analysis for several carbohydrates in fraction #2 are shown in Table 3. It was clear that these preparations contained sugars, uronide and phenolic compounds.

Campbell and Labavitch (1991a) reported that pectic oligomers in degree of polymerization (DP) from 5 to 13 galacturonic acid, with 90% of carbohydrates in DP 7 to 9, promoted ethylene production in pear cells. We also examined the effects of purified pectic oligomers in DP 2 to 12 on ethylene production in our assay system and obtained variable results (data not shown). This implies that the cell wall component from peach that inhibits ethylene production is not simple structure as oligomers of galacturonic acid. Gross (1985) and Kim et al. (1987) reported that 400 and 800 $\mu\text{g galactose} \cdot \text{g}^{-1}$ fresh weight stimulated ethylene production in tomato fruit. However, in our experiments galactose at 600 $\mu\text{g} \cdot \text{g}^{-1}$ fresh weight of apple fruit discs did not stimulate ethylene production (Table 4). This suggests that the stimulatory effect of galactose on ethylene production may depend on the commodity. Furthermore, these workers used mature green tomato, and we used already harvested and stored apple fruit, and this may also contribute to the different response to galactose. Standard compounds (galac-

Table 1. Inhibition of ethylene production by the fractions obtained via passage through PD-10 column^z.

Incubation time in assay solution (hr)	Control	Fractions	
	(nl C ₂ H ₄ · gFW ⁻¹ · h ⁻¹)	High molecular	Low molecular
		Relative ethylene production against control (%)	
2	9.1	87 ± 3	71 ± 6
4	35.7	82 ± 1	46 ± 4
6	46.6	89 ± 1	39 ± 1
8	46.9	87 ± 10	27 ± 1

^z AIS digested by pectolyase Y-23 was applied to PD-10 column, and the resultant fractions were passed through a glass filter and added to the assay solution (see Methods).

Table 2. Effect of dialysis of the AIS extracts after pectolyase Y-23 digestion on ethylene production by apple discs.

Incubation time in assay solution (hr)	Control	Treatment	
	(nl C ₂ H ₄ · gFW ⁻¹ · h ⁻¹)	Dialysis	Without dialysis
		Relative ethylene production against control (%)	
2	17.9	107 ± 6	31 ± 2
4	52.9	105 ± 2	18 ± 0
6	61.8	118 ± 19	14 ± 2
8	66.3	96 ± 12	8 ± 1

Fig. 2. Inhibition of ethylene production by the fractions recovered following TLC plate.

A ; control.
B ; #1.
C ; #2.

Table 3. Analysis of peach digest fraction # 2².

	Content	
Total sugar ($\text{mg} \cdot \text{ml}^{-1}$)	7.9 $\pm$ 0.4	(as glucose)
Uronic acid ($\mu\text{g} \cdot \text{ml}^{-1}$)	39.9 $\pm$ 0.6	(as galacturonic acid)
Flavanol tannin ($\mu\text{g} \cdot \text{ml}^{-1}$)	94.5 $\pm$ 26.0	(as catechin)
Phenol ($\mu\text{g} \cdot \text{ml}^{-1}$)	636.5 $\pm$ 4.9	(as catechin)
Chlorogenic acid ($\mu\text{g} \cdot \text{ml}^{-1}$)	40.0 $\pm$ 0	(as chlorogenic acid)

² The following methods were employed : anthrone (Morris, 1948), carbazole (Dische, 1947), Vanillin- H_2SO_4 method (Sondheimer, 1953), Folin-Denis (Folin and Denis, 1915), diazo method (Nakabayashi and Ukat, 1963).

turonic acid ; 100, 10 and 1 mg/assay : glucose ; 300, 30 and 3 mg/assay : mannose, fucose, galactose, rhamnose, xylose and arabinose ; 0.6 mg/assay) were also tested in our assay system (Table 4). Except for galacturonic acid at 100 mg,

we did not observe any distinct inhibitory effect on ethylene production. Galacturonic acid at 100 mg inhibited ethylene production by 72% during the 8 hr incubation. From the data in Table 3, the galacturonic acid component in fraction # 2 was estimated to be about $40 \text{ mg} \cdot \text{ml}^{-1}$. However, comparative analysis following addition of galacturonic acid to fraction # 2 on dilution (data not shown) indicated that the inhibitory effect of fraction # 2 was not due to the high concentration of galacturonic acid.

The supernatant from AIS fraction was slightly acidic with a pH in the range of 5.3 to 4.6 after 8 hr incubation. Neither malic acid nor citric acid inhibited ethylene production in our system (Table 5). It was apparent, therefore, that the ethylene inhibitory substance(s) was different other than these two and its effect was not as a consequence of acidic pH. Although TLC analysis of fraction # 2 utilizing the *p*-toluenesulphonic acid method (Roux, 1957) and diphenylamine-aniline method (Bailey and Bourne, 1960) of detection revealed the presence of hexose and phenolic compounds, these compounds existed separately and not as the aglycon (data not shown).

We also examined whether peach fruit can release the inhibitory substance(s) without pectolyase Y-23 digestion. AIS prepared from unripened and ripened peach fruits were resuspended in distilled water, and the extract obtained after 24 hr was directly used for assay of ethylene production. There was no inhibitory effect on ethylene production by the AIS fraction from unripened fruit. However, the fraction from ripened fruit partially inhibited ethylene production (Fig. 3). To examine for possible autocatalytic effects in the peach fruit itself, AIS was incubated with freshly

Table 4. Effect of various carbohydrates on ethylene production by apple discs.

Carbohydrate	Concentration (mg/assay)	Incubation time in assay solution (hr)			
		2	4	6	8
Relative ethylene production against control (%)					
Galacturonic acid	1	129 ± 1	109 ± 4	103 ± 19	99 ± 12
	10	130 ± 2	101 ± 1	99 ± 3	78 ± 1
	100	120 ± 1	83 ± 2	72 ± 5	72 ± 0
Sucrose	40	137 ± 6	122 ± 10	140 ± 15	133 ± 10
	400	113 ± 3	114 ± 9	128 ± 0	126 ± 3
Glucose	3	106 ± 5	112 ± 5	120 ± 4	97 ± 15
	30	115 ± 20	98 ± 18	109 ± 10	94 ± 1
Fructose	3	104 ± 6	114 ± 10	86 ± 1	95 ± 3
	30	112 ± 10	113 ± 13	103 ± 9	103 ± 4
Sorbitol	1	85 ± 12	86 ± 5	99 ± 5	98 ± 12
	10	111 ± 12	93 ± 11	104 ± 6	102 ± 2
Inositol	1	87 ± 9	97 ± 4	104 ± 7	114 ± 4
	10	80 ± 4	91 ± 8	101 ± 1	118 ± 1
Mannose	0.6	87 ± 18	91 ± 8	98 ± 15	94 ± 20
Fucose	0.6	102 ± 5	73 ± 19	91 ± 3	90 ± 1
Galactose	0.6	102 ± 7	81 ± 1	86 ± 4	83 ± 12
Rhamnose	0.6	98 ± 17	96 ± 14	92 ± 15	93 ± 11
Xylose	0.6	105 ± 3	101 ± 7	108 ± 2	110 ± 6
Arabinose	0.6	115 ± 0	89 ± 9	76 ± 28	88 ± 10
Maltose	3	114 ± 19	117 ± 15	114 ± 15	107 ± 19
	30	111 ± 17	107 ± 7	75 ± 17	104 ± 7
	150	99 ± 2	99 ± 10	98 ± 13	87 ± 9

Table 5. Effect of organic acids and pH on ethylene production by apple discs.

Organic acid	Concentration (mg/assay)	Incubation time in assay solution (hr)				pH ^z	
		2	4	6	8	Initial	Final
Relative ethylene production against control (%)							
Malic acid	1	45 ± 2	127 ± 1	115 ± 3	93 ± 11	4.9	5.2
	10	77 ± 17	86 ± 13	94 ± 9	88 ± 12	3.3	3.4
Citric acid	1	140 ± 14	126 ± 9	107 ± 13	78 ± 9	5.0	5.2
	10	114 ± 29	121 ± 4	94 ± 2	68 ± 2	3.4	3.5

^z pH was assessed before incubation (initial) and after incubation for 8 hr (final). Data from average of 2 samples.

prepared peach fruit homogenate instead of pectolyase Y-23 digest. Addition of the resulting supernatant to the assay solution inhibited ethylene production by about 50% after a 20 hr incubation. By contrast, addition of the supernatant following treatment with boiled peach fruit homogenate inhibited ethylene production only slightly (16%) (Table 6). These data indicate that peach fruit may release the inhibitory substance(s) from AIS, although not effectively as pectolyase Y-23 digestion.

Thus, a cell wall component in peach fruit may have a physiological role on ripening and senescence of peach fruit via regulation of ethylene production. Once the structure of the substance(s) is determined, it may be possible to elucidate the key enzyme(s) that are likely to play a regulatory role.

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Fig. 3. Effect of the cell wall extracts from peach fruit of different ripeness on ethylene production by apple discs^z.

A ; control. B ; unripened peach fruit. C ; ripened peach fruit.

^z AIS from pre- and post-maturing peach fruit was resuspended in distilled water and passed through a glass filter. The resultant filtrate was added to the assay solution.

Table 6. Effect of AIS extracts digested autocatalytically by peach fruit homogenate on ethylene production by apple discs.

Treatment	Digestion time with peach fruit homogenate (hr)		
	2	9	20
Relative ethylene production against control (%) ^z			
No boiling	99 ± 15	81 ± 4	50 ± 3
Boiling	81 ± 7	77 ± 8	84 ± 8

^z Data from 8 hr incubation in assay solution.

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リンゴ果実切片の ACC 依存エチレン発生を抑制するモモ細胞壁画分について

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摘 要

モモ果実細胞壁画分に、リンゴ果実切片の ACC 依存エチレン発生を抑制する物質を見いだしたので、その諸性質を検討するとともに部分精製を試みた。

1. 軟化前後のモモ果実を 80% エタノール抽出し、アルコール不溶性固形物 (AIS) 画分を得た。AIS をベクトリアーゼ Y-23 とともに蒸留水に再懸濁し、酵素消化後、その上清をエチレン測定に供試したところ、著しいエチレン発生抑制効果が認められた。

2. ベクトリアーゼ Y-23 処理後の上清を PD-10 カラムに通すと、エチレン発生抑制効果は低分子画分 (6~12 ml) において見いだされた。さらに、ベクトリアーゼ Y-23 処理後の上清を透析すると抑制効果が消失した。このためこの物質の分子量は少なくとも 3,000 以下であることが推定された。

3. エチレン発生抑制効果の認められた低分子画分を薄層クロマトグラフィー (TLC) で展開すると、低い R_f 値においてエチレン発生抑制効果が認められた。続いて #1 (R_f=0.24) および #2 (R_f=0.44) の 2 つの画分について TLC プレートからの回収を行った。これら 2 つの画分では、エチレン発生抑制効果は #2

において認められた。この #2 画分には糖、ウロン酸そしてフェノール様物質が含まれていたが、配糖体としては存在していないことが明らかとなった。

4. エチレン発生抑制効果がモモ果実の AIS において認められたことより、生体でのこの物質の働きについて調べた。軟化前後モモ果実の AIS を水抽出し、その上清をエチレン測定に供試した。軟化前の果実の AIS からの水抽出画分にはエチレン発生抑制効果が認められなかったが、軟化後果実の AIS からの水抽出画分にはエチレン発生抑制効果が認められた。さらに、モモ果実の磨砕物 (モモ果肉水抽出後の残渣画分) で AIS を消化後の上清をエチレン測定に供試したところ、エチレンの発生は対照区の約 50% となった。モモ果肉を煮沸した後、同様に処理したところ、エチレンの発生抑制効果が認められなかった。

これらより、モモ果実の AIS に低分子のエチレン発生抑制物質が存在していること、またモモ果実自体にその物質を作り出す能力のあることが明らかとなった。この物質は AIS (果実細胞壁) 由来と考えられるため、モモ果実の成熟・老化過程においてこの物質が何らかの調節機能を果たしている可能性が示唆された。

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