Revista Ceres 28(159):435-443. 1981.

SPECIFIC INHIBITION OF PECTINMETHYLESTERASE ISOZYMES BY ETHYLENE DURING TOMATO FRUIT RIPENING [1]

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1. INTRODUCTION

Most studies of fruit softening have focussed on the role of pectinmethylesterase (PME) and polygalacturonase (PG) in this process. In previous studies, multiple forms of pectinmethylesterase (PME) from tomato fruits were detected using conventional enzyme assays, gel filtration and eletrophoresis (*9*). Data presented also showed that a concentration of 100 μ 1/liter ethylene in the atmosphere above the enzyme had a stabilizing effect on PME at 6°C while it apparently suppressed PME activity at 25°C, and that tomato fruit disks treated with ethephon had a similar effect.

The physiological significance of the occurrence of more than one PME in tomatoes may be related to the complexity of pectin and the variety of changes it undergoes during fruit development and ripening (*13*). This paper describes the effect of ethylene on the enzymatic profile of PME and the enzymatic activity changes of PG caused by ethylene.

2. MATERIALS AND METHODS

Tomato plants, cv. Veegan, were grown in the greenhouse. The plants were trained to one stem and two flowers of each cluster were hand-pollinated and tagged. Remaining flowers were excised. The physiological stage of development

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of the fruits was calculated as the number of days after pollination (*5*). One hundred percent of development was taken as that time when the fruit reached its maximum size and ripening was initiated.

Fruits at 90 percent of development were divided into groups of 12 fruits each and each sample was enclosed in a 250mm diameter dessicator kept at 20-21°C. The control sample was ventilated continuously with humidified air. A mixture of 5 μ l/liter ethylene in air was applied to another sample at the rate of 1 μ l/h/100 g fruit tissue. The gas mixture was prepared according to SALTVEIT and DILLEY (*15*). A third sample was maintained in a dessicator at a constant hypobaric pressure (0.20 bars) and ventilated continuously with air saturated with water vapor (*19*).

Fruits were taken at several intervals after treatment. Pericarp discs (II mm diameter) were extracted with acetone: hexane mixture (4:5, v/v) and the pigment concentration was determined spectrophotometrically as described by MIZRAHI *et alii (10)*.

PME activity was determined as described by MEDINA (9). The PG activity was measured as change in viscosity of the solution per unit time. The reaction mixture contained 1 ml of extract, 9 ml of 0,5 percent polygalacturonic acid in 0,1 M sodium acetate buffer at pH 4.5, mixed in a viscometer (Cannon 200). One unit of enzyme activity was expressed as 1 percent decrease of initial viscosity per 10 min. Total protein was determined by Lowry's Method as described by CHERRY (3).

For eletrophoretic analysis, an aliquot of 100 μg protein extract from each treatment was loaded on a polyacrylamide gel column produced according to MAURER (*6*), using system No. 8.

Each tube was exposed to a current of 5 mA at a temperature of about 8°C. Methylene blue served as a marker. The gels were fixed in a solution of 20 percent sulfosalicylic acid for 18 hs. The staining procedure took place overnight in a staining bath containing 0.25 percent of coomassie blue, 25 percent of isopropanol, 10 percent of acetic acid and water. The destaining procedure was carried out in the same bath with the same solvent but without the coomassie blue for 24 hs. The gels were scanned in a Beckman spectrophotometer at 500 nm.

3. RESULTS AND DISCUSSION

The ripening of tomatoes harvested at 90 percent of development was retarded by storage under hypobaric pressure (0,20 bars). Chlorophyll losses from the fruit were delayed (Table 1). Synthesis of lycopene and carotene of tomato fruits were also inhibited by holding the fruits at subatmospheric pressure. SALUNKHE and WU (*16*) and WU and SALUNKHE (*19*) showed that subatmospheric pressure storage, a form of controlled atmosphere with reduced atmospheric pressure at a given temperature, extended storage life of tomatoes as well as other fruits and vegetables. Fruits stored under continuous ventilated humidified air showed normal development of lycopene and carotene and normal degradation of chlorophyll. Fruits that were treated with ethylene during the period of storage showed and accelerated rate of degradation of chlorophyll and a more rapid synthesis of lycopene and carotene (Table 1). In tomato fruits, decreases in chlorophyll and increases in lycopene and carotene accompany the ripening process (*14*). The inhibition of these changes, under hypobaric pressure or the acceleration by ethylene application, can be attributed to the inhibition and acceleration of the ripening processes of the tomato fruits, respectively.

The effects of ethylene and hypobaric conditions on the activity of PME and PG are shown in Table 2. Soluble PME was not affected by the treatments with the activity remaining low during the course of the experiment. The activity of

TABLE 1 - Changes of pigment concentration in tomato fruit by ethylene application. The statistical limits are estimates of the standard deviations of the population (n^1 = 12)

Treatment	Days after Treatment	Absorbance/g fresh weight at different wave lenghts (nm)			
		Carotene		(Lycopene)	Chlorophyll
		(444)	(470)	(502)	(661)
Control	2	3.09±0.21	1.66±0.07	1.92±0.02	1.16±0.07
	4	8.43±0.38	1.23±0.06	2.61±0.29	0.59±0.05
	6	11.99±0.72	2.01±0.22	11.13±1.69	0.33±0.05
Ethylene (5 µl/l)	2	8.49±0.47	6.31±0.46	4.94±0.42	0.59±0.12
	4	11.73±1.29	11.62±1.52	8.62±1.35	0.26±0.02
	6	13.42±0.21	14.34±1.64	13.19±1.82	0.16±0.04
Hypobaric (0.20 bars)	2	2.03±0.11	1.66±0.07	1.91±0.07	1.19±0.05
	4	1.39±0.08	1.39±0.06	2.34±0.06	1.13±0.07
	6	1.26±0.08	0.99±0.03	3.37±0.19	0.82±0.19

TABLE 2 - Effect of ethylene on the activity of pectolytic enzymes of tomato fruits. The statistical limits are estimates of the standard deviations of the population (n^1 = 12)

Treatment	Enzyme activity units/mg protein	Days after treatment		
		2	4	6
Control	Soluble PME	3.8±0.6	4.2±0.6	5.3±0.7
	Insoluble PME	61.9±1.6	79.0±2.3	67.3±2.7
	PG	14.1±0.8	15.5±1.1	30.7±2.2
Ethylene (5 µl/1)	Soluble PME	4.2±1.6	3.7±1.2	4.5±0.8
	Insoluble PME	23.9±2.1	43.2±1.7	46.7±3.4
	PG	14.9±1.4	40.9±2.1	40.8±2.3
Hypobaric (0.20 bars)	Soluble PME	3.7±0.4	3.5±0.8	4.2±0.7
	Insoluble PME	62.8±1.2	60.9±1.6	59.5±1.7
	PG	11.8±0.7	13.9±1.4	14.1±1.7

insoluble PME, which, presumably plays a more important role during the ripening process, was greatly altered by ethylene treatment. Two days after ethylene application, PME activity was inhibited when compared with the control and hypobaric treatments. Even though the activity increased after 4 and 6 days of ethylene treatment, the rate of activity was still lower than the other treatments. Control fruits showed normal development and normal increase in PME activity. At day 4, the control fruits showed maximum insoluble PME activity, consistent with the fact that, at day 4, fruits had probably attained the climacteric peak based on the fact that marked changes in pigment concentrations had occurred (Table 1). At day 6, the fruits appeared to have ripened fully and a decrease in PME activity was observed. Hypobaric treatment did not alter the activity of PME, nor were any pigment or PG changes observed.

The pattern of PG activity during the experiment was different from that of PME. The control fruits showed an increase in PG activity until day 6. Fruits treated with ethylene had attained maximum activity by day 4. This is consistent with the fact that ethylene can accelerate the ripening process by increasing lycopene synthesis (7). HOBSON (4) found a many-fold increase in PG activity of tomato fruits as they progressed from the green stage to the red stage. Fruits treated with ethylene had already attained red color at day 4 (Table 1).

The resolution by scanning the gel in a spectrophotometer is shown on Figure 1. Fruits harvested at 90 percent of development and stored for 2 to 6 days at

subatmospheric pressure of 0.20 bars showed the presence of three PME isozymes independent of time of sampling. The same pattern was obtained from control fruits. In both cases, one major isozyme was predominant. These data are consistent with the finding that three PME isozymes are already present in fruits at 90 percent of development (*13*) and do not change with further development (*9*).

A different picture was obtained when fruits received ethylene treatment. Only one isozyme could be detected at days 2 to 6 under treatment with ethylene. This isozyme corresponded to that which appeared to be the major isozyme in both control and hypobaric-treated fruits.

One common way to demonstrate the mechanism of action of a plant hormone is to assume that the hormone may interact with some cellular component of macromolecular nature. While recent findings circumstantially indicate that ethylene may operate as a hormone through this mode (*11, 12*), there is no direct supporting evidence. Ethylene has long been known to be involved in fruit ripening (*1, 2*). Doubt has now been raised that the role of ethylene in the ripening of tomato fruits is a primary one. McGLASSON *et alii* (*8*) proposed that ethylene production is an integral part of ripening but that it does not act as a trigger. SIMONS and BRUINSMA (*17*) suggested that the presence of a substance, not involved in ethylene synthesis, is required in the pericarpo of tomato fruit for ripening to occur. The data presented here lead us to assume that the PME isozyme complex may play an important role in initiating the ripening process. The interaction of ethylene and PME molecule could be stipulated by the fact that in both *in vitro* and *in vivo* systems exogeneous ethylene caused apparent suppression of PME enzyme activity.

The effect of ethylene on PME can be explained in two ways:

1. Ethylene effects stabilization of one or more isozymes of PME. The two fractions (fractions III and IV in Figure 1) appearing in fruit extracts throughout development could be just one fraction. During the process of extraction, certain manipulation(s) could bring about the structural change of enzyme causing its dissociation into more than one form. Ethylene may act as a stabilizing agent to prevent such dissociation. When exogenous ethylene was applied to tomato fruit, the presence of only one band could be the native form of PME in the fruit.

2. Ethylene treatment may effect preferential suppression of the PME isozymes. Exogenous application of ethylene caused suppression of fraction II (Figure 1), or suppression of fractions II and IV if fraction IV was not an artifact. Whether ethylene caused inhibition of *de novo* synthesis of PME isozyme complex is not known. Although abundant information is available with regard to the involvement of PME on softening of fruits, no role in the initiation of ripening has been ascribed to PME. Traditionally, ethylene has for some time been considered as the prime causal agent in fruit ripening. However, studies using propylene as an active analogue of ethylene showed that although the ripening of fruit was advanced in time, such ripening was abnormal (*9*).

We suggest that the onset of ripening in normal tomato fruit is not controlled solely by endogenous ethylene but rather that the initiation of ripening involves a breakdown in cellular organization. During growth of tomato fruits, the major forms of PME are fractions III and IV which may act specifically on long chain pectic molecules. The hydrolysis of the ester groups may be an important factor in the alteration of cell walls. Prior to the respiratory climacteric, increases in PG activity may cause an increase in short chain pectic molecules. Presumably, a specific isozyme of PME (Fraction II) is synthesized to handle these molecules. Synthesis of this specific PME isozyme is likely to precede the burst in ethylene production and other concomitant ripening changes and may be intrinsic to the breakdown of organization resistence and the induction of ripening (*18*).

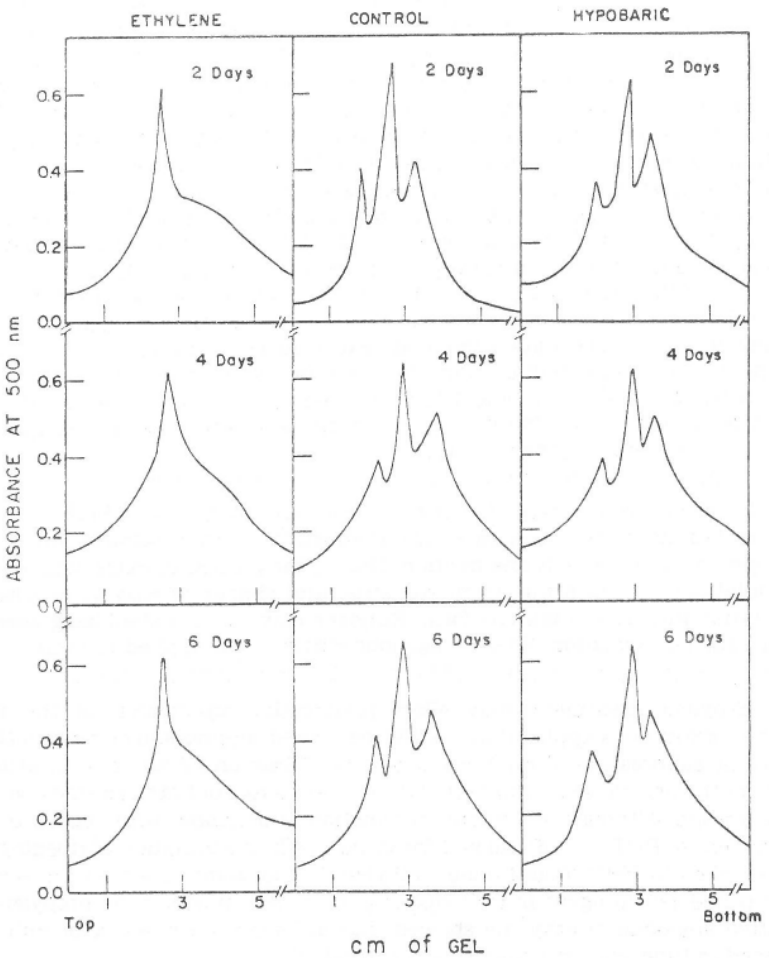

FIGURE 1. Eletrophoresis resolution of PME extracts from tomato fruits exposed to 5 µl/l ethylene and hypobaric (0.2 bars) conditions.

Subsequent increases in the endogenous ethylene level may act to stabilize the PME isozymes, but this would have no further effect on ripening changes.

4. SUMMARY

In this paper, experiments and data are presented pertinent to the effect of ethylene on the enzymatic activity of both pectinmethylesterase (PME) and polygalacturonase (PG) and ethylene effects on the enzyme profile of PME during tomato fruit ripening.

Tomato plants, cv Veegan, were grown in the greenhouse. Fruits were picked at 90% of development and enclosed in a dessicator kept at 20ºC. The control sample was ventilated continuously with humidified air. A mixture of 5 μl/liter ethylene in air was applied to another sample. The third sample was mantained in a dessicator at a constant hypobaric pressure (0.20 bars). Fruits were analyzed at several intervals after treatments. Pigment concentration was determined spectrophotometrically. The individual samples were assayed for PME and PG activities and the PME extracts were further developed by a disc eletrophoresis.

The ripening of tomatoes was retarded by storage under hypobaric pressure. Fuits that were treated with ethylene showed an accelerated rate of degradation of chlorophyll and a more rapid synthesis of carotene and lycopene. The PG activity of tomato fruits under ethylene treatment increased as they progressed from the green stage to the red stage. The PME activity was inhibited by ethylene. This inhibition may be explained by the preferencial suppression of PME isozyme fractions II and IV.

5. RESUMO

Este trabalho teve dois objetivos: 1.º) estudar a ação específica ou preferencial do etileno sobre as frações isoenzimáticas da pectinametilesterase, (PME), 2.º) estudar o efeito deste hormônio sobre a atividade da poligalacturonase (PG) durante o processo de maturação dos frutos de tomateiro.

Frutos de tomateiro, cultivar Veegan, foram produzidos em casa-de-vegetação e colhidos quando apresentavam 90% do seu desenvolvimento total. Após a colheita, os frutos foram submetidos a três sistemas de armazenamento. Considerou-se como controle quando o ambiente apresentava saturado com vapor d'água. Hipobárico quando se reduzia a pressão do sistema para 0,20 bars e atmosfera controlada quando o etileno era aplicado constantemente no sistema numa concentração de 5 μl. Durante o armazenamento os frutos foram amostrados e analisados quanto à composição de clorofila e pigmentos carotenóides e, paralelamente, foram efetuadas análises da atividade da PME e PG, tendo a PME em adição sido fracionada por eletroforesis.

Verificou-se que o armazenamento hipobárico não permitiu qualquer alteração na maturação dos frutos evidenciados pela estabilidade das concentrações dos pigmentos e das enzimas PME e PG. Entretanto, o tratamento com etileno contribuiu para uma degradação mais rápida de clorofila e uma síntese mais acentuada dos carotenóides quando comparados com o controle. A PG teve sua atividade aumentada à medida que o fruto atingia maior grau de maturação. A atividade da PME, fase insolúvel, foi inibida pelo etileno. Esta inibição foi explicada pela ação preferencial do etileno sobre as frações isoenzimáticas de número II e IV, necessárias para a manifestação do seu modo de ação.

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