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Ethylene Production by Apple Protoplasts

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ABSTRACT

Freshly prepared protoplasts from apple tissue that produced ethylene were obtained. Ethylene production was inhibited by osmotic shock, 0.01% Triton X-100, and aminoethoxyvinyl glycine. Protoplasts as well as the ethylene system were not greatly affected by protease treatment.

Most plant systems produce ethylene from methionine (6) and this production is sensitive to osmotic shock (2, 5, 9). Even though the *in vivo* substrate is known, no one has reported the isolation of an *in vitro* enzyme system from plant tissues capable of converting methionine to ethylene. Apparently the system does not survive cell destruction. When ethylene-producing tissues are disrupted, their ethylene production is greatly reduced or eliminated depending upon the degree of cell destruction. Such an effect on ethylene synthesis occurred even under the relatively mild conditions of protoplast production (9). The evidence to date indicates that the ethylene system is associated with the plasma membrane (5, 8, 10) or perhaps with a plasma membrane-cell wall complex (9).

We undertook to identify further the site of ethylene production in apple tissue and to characterize the ethylene-forming system. The data we present show that naked protoplasts, with no cell wall whatever, produce ethylene.

MATERIALS AND METHODS

Source of Reagents. Most reagents except for Cellulysin[1] (Calbiochem), Driselase (Kyowa Hakke Kogyo, New York) Rhozyme HP-150 (Rohm and Haas), methanol (Burdick & Johnson), Pronase, chymotrypsin, trypsin, chymopapin (Sigma), Proteinase K (Beckman), Fungizone (Flow Laboratories), and L-[3,4-^{14}C]methionine (Research Products International Corp.) were from local suppliers and of reagent grade.

Cellulysin, Driselase, and Rhozyme HP-150 were desalted by ultrafiltration with an Amicon PM-10 membrane, and stored frozen for subsequent use. Desalting removed about 60% of the original dry weight of the Cellulysin preparation. All solutions were sterilized by filtration (Millex or Millipore HA) prior to use.

Protoplast Isolation. Golden Delicious apples from 0 C storage or commercial sources were disinfected by immersion in 70% (v/v) ethanol for 1 min followed by immersion in 1.25% sodium hypochlorite for 5 min. The apples were then rinsed three times with sterile H_2O and thinly sliced with a sterile vegetable slicer. Discs were punched from the slices with a sterile 1-cm-diameter cork borer and immediately placed in sterile basal medium (0.8 M sorbitol, 10 mM Mes [pH 6.0], 0.1 M $CaCl_2$, 10 μg/ml chloramphenicol, and 5 μg/ml Fungizone). When sufficient discs were collected, the collection requiring less than 1 h, 4 to 5 g of discs were added to 10 ml incubation medium (basal medium plus cell-wall-digesting enzymes) contained in plastic Petri dishes.

Protoplasts were isolated by filtration of the tissue, macerated in the incubation medium, with a 140-μm mesh stainless steel screen. In later experiments, the medium-macerated tissue was filtered first with sterile glass wool and then the screen. The glass wool effectively removed most of the large pieces of tissue. Protoplasts were allowed to settle for ~15 min before the supernatant was aspirated. Protoplasts were resuspended two or three times in basal medium and collected as above. After the final washing, the protoplasts were monitored for ethylene production in basal medium.

Ethylene Production. Protoplasts suspensions were placed in sterile 5-ml vials or 25-ml Erlenmeyer flasks along with other additions, and the vessels stoppered with sterile serum caps. Gas samples were taken at intervals, after which each vessel was flushed with at least 3.5 times its volume of sterile air. Ethylene was measured by GC (7). To determine if methionine serves as an ethylene precursor, protoplasts were incubated with L-[3,4-^{14}C]-methionine (53 mCi/mmol). Ethylene was trapped (in mercuric acetate) by transferring, after various periods of incubation, the gases in the incubation vial to an evacuated scintillation vial containing 2 ml 0.1 M mercuric acetate in methanol through an argyle extension tube with No. 22 Luer-slip needles on both ends (4). The remaining gases were swept into the scintillation vial when the vacuum in the incubation vial was released with a syringe needle. Three h after the transfer of the gases, the scintillation vials were opened, and 10 ml Aquasol liquid scintillation fluid were added.

After transferring the gases from the incubation vial or flask, the protoplast suspension was made to 10% (w/v) trichloroacetic acid and placed in an ice bath for at least 30 min before the acid-insoluble material was collected by filtration on Whatman GF/C glass filters. Radioactivity in the acid-insoluble material and in ethylene was determined in 5 ml Liquifluor or 10 ml aquasol scintillation fluid, respectively.

RESULTS

Protoplast Isolation. Several hydrolytic enzymes were tested in the isolation of protoplasts. When incubated with a combination of Cellulysin, Driselase, and Rhozyme HP-150, in a solution of 0.6 M sorbitol, 100 mM $CaCl_2$, and 10 mM Mes (pH 5.0), apple tissue yielded good quantities of protoplasts within 4 h. However, these did not produce significant levels of ethylene. We found that like pectinase (9), Rhozyme HP-150 inhibited ethylene production of apple discs, except that it did not macerate the tissue. We found that overnight digestion of tissue with Cellulysin (11) in 0.8 M sorbitol, 100 mM $CaCl_2$, and 10 mM Mes (pH 6) produced a good yield of protoplasts which formed ethylene. These protoplasts

[1] Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the United States Department of Agriculture and does not imply its approval to the exclusion of other products that may also be available.

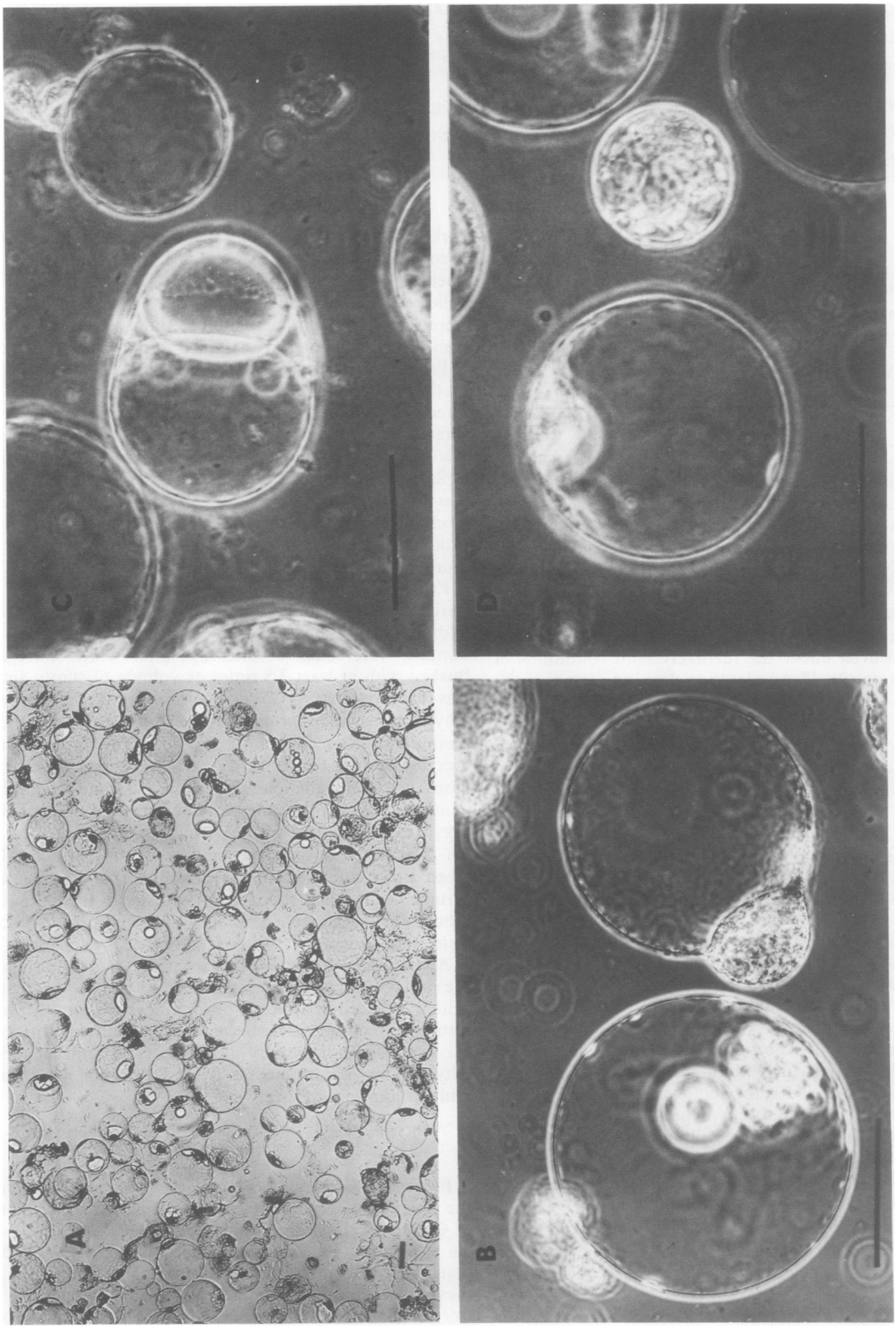

FIG. 1. Light micrographs of apple tissue protoplasts. Bar line on each figure represents 100 μm. Micrograph A was taken with bright field illumination; micrographs B, C, and D were taken with phase contrast.

Table I. Effect of $CaCl_2$ concentration on ethylene and protoplast production.

Thin Golden Delicious apple slices (0.5 gm) were incubated in 3 ml of 0.8 M sorbitol containing 10 mM Mes (pH 6.0), 30 µg chloramphenicol, 15 µg fungizone, 150 mg Cellulysin and various concentrations of $CaCl_2$. Ethylene production was monitored periodically.

Treatment	Incubation (h)					
$CaCl_2$ Concn(mM)	0-2	2-4	4-6	6-11	11-23[2]	25-27
	Ethylene production % of control[1]					
0 (Control)	100	100	100	100	100	100
1	116	138	165	266	257	179
3	117	159	242	413	407	218
10	115	161	270	561	703[3]	318
30	102	148	273	663	1231[3]	556
100	102	141	276	824	1460[3]	716
300	91	133	239	624	1223	855

[1]Control readings were 84.3, 48.5, 18.8, 6.0, 0.9 and 0.8 nl/g/h for readings at 0-2, 2-4, 4-6, 6-11, 11-23 and 25-27 h, respectively.

[2]All tissues were well macerated by 23 h except for the control and 300 mM $CaCl_2$

[3]Contain large protoplasts with large central vacuole.

Table II. Effect of different proteases, Triton X-100 and aminoethoxyvinyl glycine (AVG) on ethylene production of apple protoplasts

Treatment	Incubation (h)				
	0-1	1-2	2-3	0-2	2-4
	Experiment 1			Experiment 2	
	nl C_2H_4/h				
Control	15.3	11.1	10.0	13.7	8.3
Trypsin	12.4	12.4	9.2	14.2	8.5
Proteinase K	13.1	13.2	13.0	14.0	10.6
Chymotrypsin	12.2	10.3	7.8	13.5	7.8
Pronase	8.3	8.7	8.8	9.7	9.8
Chymopapain	11.5	10.3	9.2	13.2	8.1
0.001% Triton X-100	11.9	11.8	10.3	12.6	6.9
0.01% Triton X-100	9.5	4.3	1.3	7.0	2.8
AVG 1 mM	8.2	2.7	1.9	-	-
0.1 mM	-	-	-	4.1	1.4

All proteases were added to protoplast suspensions at 1 mg/ml final concentration. Each value is the average of duplicates.

were free of obvious cell wall material as determined by: (*a*) lack of cell wall structure observed at the light microscope level; and (*b*) absence of fluorescence associated with the protoplast caused by Calcofluor white. Most of these protoplasts (Fig. 1, A and B) were between 188 and 225 µm in diameter, had a localized concentration of cytoplasm, and had a large vacuole. Some had multiple vacuoles (Fig. 1C) and some had small vacuoles extending from the protoplast. Others were smaller (50–85 µm) and more dense, with no apparent vacuole (Fig. 1D). Ethylene production appeared to be associated with the large protoplasts.

Effects of $CaCl_2$ on Ethylene Production. The ethylene-synthesizing system of preclimacteric apple tissue was previously shown to be stabilized or more active if $CaCl_2$ was present in the incubation medium (3). We found with postclimacteric fruit discs that 100 mM $CaCl_2$ was about optimum for maintaining ethylene production. The effects of $CaCl_2$ on preserving the ethylene-synthesizing system as well as on preventing browning of tissues were particularly obvious after long incubation periods of 10 to 24 h. The rate of ethylene production by tissues incubated with Cellulysin in the absence of $CaCl_2$ declined about 99% over 27 h, and tissue maceration and protoplast production were minimal (Table I). Adding 1 mM $CaCl_2$ helped prevent the loss of ethylene synthesis, and 100 mM $CaCl_2$ appeared close to the optimum concentration. This concentration of $CaCl_2$ also was the optimum for protoplast production. Higher concentration (300 mM) inhibited tissue maceration. Ca appeared to have a triple function: (*a*) to accelerate cell wall disintegration in the 1 to 100 mM range; (*b*) to stabilize protoplasts; and (*c*) to stabilize the ethylene-forming system.

Effect of Proteases. Protoplasts were treated with various pro-

Table III. Effect of osmotic shock and aminoethoxyvinylgycine (AVG) on ethylene production of apple protoplasts

Treatment	Incubation (h) 0-2	2-4
	nl/h	
None	3.6	3.0
0.4 M sorbitol - 50 mM $CaCl_2$	0.5	0.0
0.4 M sorbitol - 100 mM $CaCl_2$	0.9	0.0
0.1 mM AVG	2.8	2.2

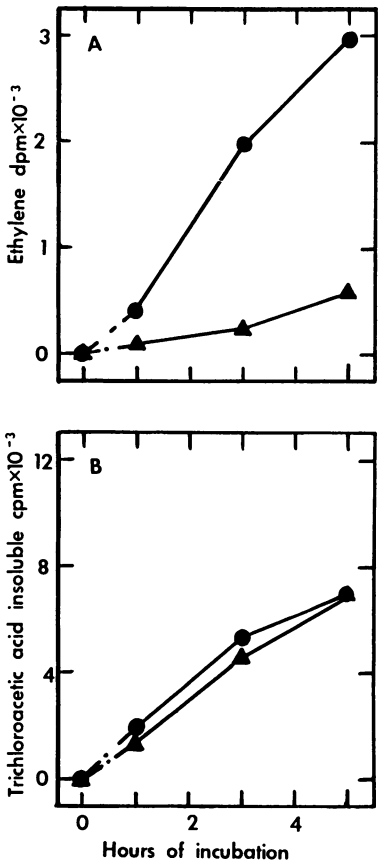

FIG. 2. Metabolism of L-[3,4-^{14}C]methionine to C_2H_4 (A) and trichloracetic-acid-insoluble material (B) by apple protoplasts. One ml of protoplasts (26.25×10^{-4}) was incubated in 0.25 μCi L-[3,4-^{14}C]methionine (53 mCi/mmol) with (▲) and without (●) 100 μM AVG. Ethylene was trapped in 0.1 M mercuric acetate in methanol (4). After collecting the ethylene, the protoplast suspension was made to 10% trichloroacetic acid and the acid-insoluble material was collected by filtration on glass filters.

teases in attempts to determine the effect of the proteases on the stability of ethylene production and on protoplast structure (Table II). Pronase was the only protease tested that lowered the rate of ethylene synthesis, but that lowered rate was maintained with increasing incubation time; whereas, the rate for control protoplasts declined. Pronase, after inducing the initial inhibition, and Proteinase K appeared to retard slightly the decline in ethylene formation of protoplasts. None of the proteases appeared to affect the stability of the protoplasts as determined by microscopic examination.

Inhibition of Ethylene Production. Ethylene production by most plant tissues, especially apple slices, is inhibited by AVG[2] (7). The effect of AVG on total ethylene production of protoplasts was variable. In some experiments there was good inhibition of ethylene production (Table II), whereas in others, there was little inhibition (Table III). In all protoplast experiments with L-[3,4-^{14}C]methionine, AVG inhibited [^{14}C]ethylene production, even when total ethylene production was little affected (Fig. 2). Our apple protoplasts were also capable of incorporating methionine into trichloroacetic-acid-insoluble material.

Ethylene production was greatly inhibited when protoplasts were given an osmotic shock by dilution of the sorbitol concentration to 0.4 M with either water or 100 mM $CaCl_2$ (Table III), or when they were lysed with detergent (Table II). Diluting the sorbitol concentration to 0.4 M with water or 100 mM $CaCl_2$ caused lysis of most of the protoplasts, as observed microscopically. Triton X-100 at 0.01% lysed almost all of the protoplasts, but 0.001% had little effect.

DISCUSSION

By the use of a proper concentration and combination of cell-wall-digesting enzymes, osmolarity, pH, and $CaCl_2$, freshly harvested apple protoplasts were obtained which synthesized ethylene. To do this, we used cell-wall-digesting enzymes that contain no or low levels of inhibitory materials like those contained in pectinase (9) and Rhozyme HP-150. A good method for preparing protoplasts from postclimacteric Golden Delicious apples routinely was to incubate the tissues overnight in a digestive solution of 0.8 M sorbitol, 10 mM Mes (pH 6.0), 100 mM $CaCl_2$, and 5% Cellulysin. The ethylene-generating system of these protoplasts decayed after isolation. The cause of this decay was probably related to aging of the protoplasts and needs further investigation.

Our protoplast preparations were metabolically active, as shown by their metabolizing L-[3,4-^{14}C]methionine to [^{14}C]ethylene and to trichloroacetic-acid-insoluble material (protein synthesis). AVG at 0.1 mM substantially inhibited the accumulation of [^{14}C]ethylene but not ^{14}C in acid-insoluble material. Although this concentration of AVG inhibited amino acid incorporation into apple slices (unpublished data), it did not inhibit incorporation by protoplasts.

The differences in ethylene production between these protoplasts and those produced earlier (9) were substantial. The freshly prepared protoplasts of Mattoo and Lieberman (9) did not produce significant amounts of ethylene, possibly because of the inhibitory nature of the pectinase and the Rhozyme HP-150 in which the protoplasts were prepared and because of the lack of $CaCl_2$. Their protoplasts had to be cultured several days before they produced ethylene. The increase in ethylene production with culture time was correlated with cell wall synthesis. This regenerated ethylene

[2] Abbreviation: AVG: aminoethoxyvinyl glycine (formally called ethoxyanalog of rhizobitoxine).

system of apple protoplasts required exogenous methionine, and was inhibited by AVG and by Pronase. In contrast, our freshly prepared protoplasts did not need exogenous methionine, but did convert L-[3,4-^{14}C]methionine to [^{14}C]ethylene and were not very sensitive to Pronase and other proteolytic enzymes, but did require a constant high concentration of Ca^{2+}.

The relative insensitivity of the ethylene-synthesizing system of some protoplasts to AVG suggests an alternate pathway for ethylene production in apples, similar to that described for tomato and avocado fruit (1). Normally, ethylene synthesis by apple tissue is very sensitive to AVG (7). Additional studies are needed to explain the significance of this AVG insensitivity in some protoplasts.

The ethylene system of our freshly prepared protoplasts was sensitive to osmotic shock and to detergents which lysed them. Although the effects of osmotic shock and detergents do not prove that the ethylene system in apple tissue is associated with membranes, they do indicate that the system needs a specific structure associated with lipids. Our data on protoplasts support evidence obtained from tissue slices and sections associating the ethylene-forming system with the plasma membrane (5, 8–10). More studies are needed to obtain direct evidence as to whether the ethylene-synthesizing system is located in the plasma membrane.

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