Phosphatidase of Sclerotinia sclerotiorum Produced in Culture and in Infected Bean

R. D. Lumsden

Research Plant Pathologist, Crops Research Division, ARS, USDA, Plant Industry Station, Beltsville, Maryland 20705.

The author thanks D. P. Andberg for assistance and for preparation of the illustrations.

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Accepted for publication 27 February 1970.

ABSTRACT

Sclerotinia sclerotiorum produces an adaptive, extracellular phosphatidase in culture on autoclaved bean hypocotyls, but little, if any, when grown in a minimal medium with 1.0 or 0.5% soybean phosphatide (lecithin) as the carbon source. Slurries of diseased bean hypocotyl tissue contained appreciable phosphatidase activity early in pathogenesis, and the activity increased with increasing symptom expression. Enzyme activity was routinely assayed by measuring the release of acid-soluble organic phosphorus (P). Maximum release of P by the enzyme from culture filtrates or infected bean tissue was at pH 4.0. Activity was low or nondetectable below pH 3.0 and above pH 6.0, and was stimulated by 0.004 M $CaCl_2$. The presence of phosphatidase in infected tissue early in disease development suggests that it may play a role in pathogenesis. Phytopathology 60:1106-1110.

Additional key words: fatty acid gas-chromatography, acyl-ester, choline analysis

A number of hydrolytic enzymes are produced by *Sclerotinia sclerotiorum* (Lib.) d By. in diseased tissue during pathogenesis, including cellulase (2, 7), polygalacturonase (2, 4) and hemicellulases (5). Presumably, these and other extracellular enzymes contribute to the progress and manifestation of the disease.

One of the very early stages in pathogenesis with *S. sclerotiorum* in bean is the production of a rapidly expanding water-soaked lesion (13). The water-soaked symptom has been attributed to a pronounced modification of plasma membranes in celery tissue infected by *S. sclerotiorum* (15). Membrane changes resulted in several-fold increases in permeability. Moreover, in this same study, the permeability change occurred well in advance of the death of cells, the invasion of cells, or the effect of pectinases on cells as detected by the ruthenium red test for pectin.

Because of their possible effect on the structure of phosphatides in host tissue membranes during pathogenesis, phosphatidases have been suggested as possible key factors responsible for changes in host cell permeability during pathogenesis (8, 16). Although *S. sclerotiorum* (*S. libertiana* Fckl.) was previously reported to produce phosphatidase when grown in a bran medium (10, 11), no discussion was made of a possible role of this enzyme in pathogenesis.

The objective of the present investigation was to confirm the ability of *S. sclerotiorum* to produce phosphatidase in culture and to attempt to associate the production of phosphatide-degrading enzymes with pathogenesis in a plant disease caused by this pathogen. Portions of this work appeared previously (6).

MATERIALS AND METHODS.—Isolate Ss-3 of *S. sclerotiorum* from bean was the principal isolate used in this investigation, and was maintained on potato-dextrose agar. Nine other isolates from several sources (Table 1) were also maintained on potato-dextrose agar.

Culture of the fungus for studies of phosphatidase production on autoclaved bean hypocotyls in a defined liquid medium and preparation of enzyme extracts from these cultures were as described for the production of cellulase by *S. sclerotiorum* (7). The procedures followed for inoculation of bean plants (*Phaseolus vulgaris* L. 'Topcrop'), with *S. sclerotiorum* were also previously described (7). Disease was rated with use of an index of zero to five. Zero indicated healthy plants; five, complete invasion and collapse of the hypocotyl tissue. Inoculated plants were harvested, and 50 1-cm portions of hypocotyl lesion area were blended in the cold with distilled water (total volume of tissue plus water = 50 ml) for 1 min in a high-speed homogenizer. The slurry was assayed for phosphatidase either directly or was strained through four layers of cheesecloth and centrifuged at 10,000 *g* for 20 min; then the supernatant fluid was assayed for phosphatidase. The pH of the supernatant was recorded.

Phosphatidase was routinely assayed by the 'cup plate' procedure of Doery et al. (3), and by measuring the release of trichloroacetic acid-soluble organic P (8). With the latter procedure, standard enzyme reaction mixtures contained 0.5% refined soybean lecithin (Nutritional Biochemicals Corp., Cleveland, Ohio), 0.05 M of succinate-NaOH buffer (pH 4.0), 0.004 M $CaCl_2$, and enzyme preparation in a total volume of 1.0 ml. After incubation for the indicated time period at 30 C, the reaction was stopped by addition of either (i) 3.0 ml 5% trichloroacetic acid and 0.1 ml 5.0% bovine serum albumin; or (ii) 0.5 ml 20% trichloroacetic acid and 0.15 ml 5.0% bovine serum albumin. These two procedures are indicated in the text as TCA extraction procedure 1 or 2. The trichloroacetic acid extract was then assayed for acid-soluble P. Enzyme activity was expressed as μg P released per ml of enzyme reaction mixture per hr, or as units of enzyme activity. One unit equals the amount of enzyme that releases 1 μg P/min, at 30 C, in the standard reaction mixture.

The release of glycerylphosphorylcholine (a result

of the removal of the 2 fatty acids from the lecithin molecule) was assayed by the method of Barron (1) with 0.5 ml of the trichloroacetic acid extract obtained from the above reaction mixture. Glycerylphosphorylcholine was estimated from a choline chloride standard curve. The precipitate obtained in the above reaction mixture, after addition of trichloroacetic acid, was assayed for acyl-ester linkages (12); and the hydrolysis of these linkages was measured after various intervals of time. Results were expresed as per cent acyl-ester bonds hydrolyzed.

The release of free fatty acids, also a measure of hydrolysis of acyl-ester linkages, was determined by gas-liquid chromatography. A reaction mixture containing 2.0 ml of 1.0% soybean lecithin in 0.1 M succinate buffer (pH 4.0), 1 ml of 0.016 M $CaCl_2$, and 1 ml *S. sclerotiorum* phosphatidase (10 mg/ml of lyophilized bean stem culture or diseased tissue extract) was incubated for 4 hr at 30 C. Fatty acids were extracted into 4 ml chloroform, applied to a 1- × 10-cm column of silicic acid (100 mesh), and eluted with 20 ml chloroform. The eluant was evaporated to dryness and the fatty acids methylated (9). Reaction mixtures containing steamed enzyme extracts, and a preparation of 0.5% lecithin saponified with 0.6 N NaOH, were treated similarly.

The samples in chloroform were analyzed in an F and M Scientific Corp., Model 810, gas chromatograph with a polyethylene glycolsuccinate, 15% gas chrom P, 100/210 mesh column (Applied Science Laboratories, State College, Pa.); column temp 170 C; detector, 250 C; injection port, 300 C; with helium at 35 ml/min as the carrier gas.

Thin-layer chromatography of the acid-soluble P products of the reaction of *S. sclerotiorum* phosphatidase with soybean lecithin was performed by the method previously described (8), except that the solvent system contained chloroform:methanol:3 M trichloroacetic acid:H_2O in a ratio of 60:40:20:15.

RESULTS.—Considerable phosphatidase activity was detected by the 'cup plate' method (3) in cell-free extracts of cultures of *S. sclerotiorum* grown on autoclaved bean stems and in extracts of infected bean hypocotyls.

Effect of pH on phosphatidase activity.—Succinate-NaOH buffer (0.05 M) was used in the range of pH 3.0 to pH 6.0, and Tris [tris(hydroxymethyl) amino methane]-HCl buffer (0.05 M) in the range pH 7.0 to 9.0 to control the pH of enzyme reaction mixtures containing extracts of *S. sclerotiorum* grown on autoclaved bean stems and extracts from infected bean hypocotyls. TCA extraction procedure 1 was used. Maximum phosphatidase activity of extracts was near pH 4.0 (Fig. 1). Activity dropped off sharply at pH 3.0, and was only slight between pH 6.0 and 7.0. No activity was detected above pH 7.0.

Effect of $CaCl_2$ on phosphatidase activity.—Enzyme activity assayed at pH 4.0 was stimulated by the addition of $CaCl_2$ to the enzyme reaction mixture (Fig. 2). Maximum enzyme activity was detected when concn of $CaCl_2$ between 4 and 5 mM were present in the reaction mixture. Amounts greater than 6 mM lowered the enzyme activity. Also, at these higher levels of $CaCl_2$ a precipitate formed which was presumably a calcium complex with lecithin substrate.

Hydrolysis of soybean lecithin substrate.—The hydrolysis of refined soybean lecithin was followed with time by measuring the release of acid-soluble P, release of glycerylphosphorylcholine, and the hydrolysis of fatty acid acyl-ester linkages. All three determinations were made from the same reaction mixtures in which TCA extraction procedure 2 was used. Each of the three assays reflected a linear rate of enzyme reaction up to about 40 min (Fig. 3). In addition, the release of acid-soluble P and glycerylphosphorylcholine were closely correlated with the increase in per cent hydrolysis of acyl-ester linkages.

Chromatography of phosphatidase reaction products.

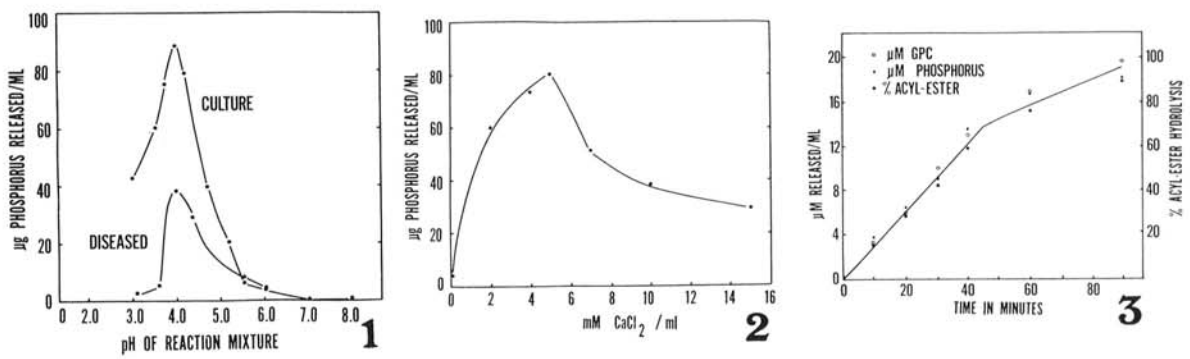

Fig. 1-3. **1)** Effect of the pH of phosphatidase reaction mixtures on enzyme activity as measured by the release of acid-soluble phosphorus. Reaction mixtures contained 0.5% soybean lecithin emulsion, 0.05 M succinate-NaOH buffer, and enzyme extract, as indicated, in a total volume of 1.0 ml. **2)** Effect of $CaCl_2$ on the phosphatidase activity in extracts of bean hypocotyl cultures of *Sclerotinia sclerotiorum*. Enzyme reaction mixtures contained 0.5% soybean lecithin emulsion in 0.5 M succinate-NaOH buffer (pH 4.0), 1.0 mg lyophilized enzyme extract, and the indicated amount of $CaCl_2$. **3)** Release of acid-soluble phosphorus and glycerylphosphorylcholine (GPC), and hydrolysis of acyl-ester linkages by phosphatidase in reaction mixtures containing extracts from *S. sclerotiorum* cultures grown on autoclaved bean hypocotyls. Reaction mixtures contained 0.5% soybean lecithin in 0.05 M succinate-NaOH buffer, 0.004 M $CaCl_2$, and 1 mg lyophilized culture extract in 1.0 ml of reaction mixture.

—Thin-layer chromatography of the trichloroacetic acid-soluble reaction products revealed that glyceryl-phosphorylcholine was released in standard reaction mixtures that contained extracts from cultures of *S. sclerotiorum* and from diseased bean tissue, but not from control reaction mixtures containing steamed enzyme extracts or extracts from healthy bean tissue. No phosphorylcholine or free choline was detected.

Gas-liquid chromatography of chloroform extracts of standard reaction mixtures incubated for 4 hr at 30 C with enzyme extract from cultures on bean stems revealed the release of fatty acids from soybean lecithin corresponding to known standards of palmitic, stearic, oleic, linoleic, and linolenic acids (Fig. 4). These same fatty acids were released in standard reaction mixtures that contained enzyme extracts from diseased bean tissue, but not in reaction mixtures that contained steamed extract or healthy tissue extract. Extracts of soybean lecithin saponified with NaOH contained these fatty acids in approximately the same amounts as extracts from the phosphatidase reaction mixture. One exception was that the fatty acid which appeared on the chromatograph after 48 min was present in low levels in the extract of the saponified lecithin, but not in the enzyme hydrolysate. The percentage of fatty acid composition of soybean lecithin was compared with the values obtained for the NaOH-hydrolyzed preparation, the phosphatidase hydrolysate, and the values reported for soybean lecithin (14) (Table 2).

TABLE 1. Phosphatidase activity of extracts of cultures of various isolates of *Sclerotinia sclerotiorum* grown on autoclaved bean hypocotyls, compared with the virulence of these isolates to Topcrop bean plants

Isolate	Source	Disease index[a] 4 Day	Disease index[a] 7 Day	Phosphatidase units/ml of extract[b]
Ss-1	New York[c] bean pod, 1966	0.3	0.7	1.5
Ss-2	New York[c] bean pod, 1967	1.2	3.5	1.4
Ss-3	Maryland[d] bean stem, 1966	3.0	5.0	0.8
Ss-4	New York[c] bean pod, 1967	2.8	4.6	1.3
Ss-5	New York[c] brussel sprouts, 1967	1.9	3.9	0.4
Ss-6	New York[c] cabbage, 1966	0.6	0.9	2.4
Ss-7	New York[c] squash, 1967	2.7	4.8	0.5
Ss-8	New York[c] pea, 1966	1.2	1.1	0.6
Ss-9	North Carolina[e] bean stem, 1968	2.2	4.5	1.7
Ss-10	Maryland[e] lima bean stem, 1968	3.2	5.0	0.2

[a] Disease index: 0 = healthy; 5.0 = all plants dead.
[b] One unit = enzyme to release 1 μg phosphorus/min at 30 C.
[c] Isolate obtained from J. J. Natti, Geneva, N. Y.
[d] Isolate obtained from R. W. Goth, Beltsville, Md.
[e] Isolated by author.

TABLE 2. Release of fatty acids from soybean lecithin by phosphatidase of *Sclerotinia sclerotiorum* compared with the release of fatty acids by saponification

Fatty acid	Fatty acid composition of soybean lecithin: Phosphatidase[a]	Saponified[b]	Reported[c]	Standard[d] fatty acids
	%	%	%	%
Palmitic (C_{16})	23.7	20.2	11.7	20.8
Stearic (C_{18})	5.9	5.6	4.0	20.0
Palmitoleic ($C_{16}=$)	0.0	0.0	8.6	
Oleic ($C_{18}=$)	9.5	8.5	9.8	20.0
Linoleic ($C_{18}2=$)	57.2	53.8	55.0	19.8
Linolenic ($C_{18}3=$)	3.9	5.9	4.0	19.5
C_{20}-C_{22}			5.5	
Unidentified	0.0	5.9		

[a] Enzyme reaction mixture contained 0.5% soybean lecithin in 0.004 M $CaCl_2$ and 0.05 M succinate-NaOH buffer at pH 4.0 and an extract of *S. sclerotiorum* grown on autoclaved bean hypocotyls. Incubation was for 4 hr at 30 C.
[b] 0.5% Soybean lecithin saponified 24 hr with 0.6 N NaOH.
[c] Literature citation 14.
[d] Standard fatty acid ester mixture (composition = 20% of each ester), obtained from Applied Science Laboratories, State College, Pa.

These values were generally in good agreement. Palmitoleic acid was the only acid previously reported (14) to be present in soybean lecithin but not detected in the NaOH or enzyme hydrolyzed preparations.

Phosphatidase production in culture.—S. sclerotiorum produced phosphatidase abundantly in cultures of the fungus grown on autoclaved bean hypocotyls (Fig. 5-A). The enzyme was most prevalent 6 days after incubation at 24 C, and in the culture liquids rather than in the culture solids. After 6 days, the activity began to decline in both the culture liquids and the culture solids.

In contrast to the relatively large quantity of enzyme produced in the natural medium, enzyme activity in the minimal medium was low and variable. Minimal medium that contained 1.0% or 0.1% soybean lecithin as a sole carbon source did not support abundant growth of the fungus, and no consistent pattern of phosphatidase production was evident. Growth was reduced even when 1.0% glucose was added to the medium containing 0.1% lecithin, as compared to a control medium that contained glucose alone. Phosphatidase activity was slight and variable when 1.0% glucose was the sole carbon source.

Phosphatidase in diseased tissue.—Difficulty was encountered with the assay of phosphatidase activity in water extracts of diseased tissue. Variability was again a problem. The enzyme activity, however, could be consistently detected in the slurry of diseased hypocotyls or in the pellet after centrifugation. Slurries of diseased hypocotyl tissue contained considerable phosphatidase 2 days after inoculation (Fig. 5-B). The activity increased with time after inoculation, and was closely correlated with the disease index.

The pH of slurries of diseased bean hypocotyls dropped from a value of pH 5.9, 1 day after inocu-

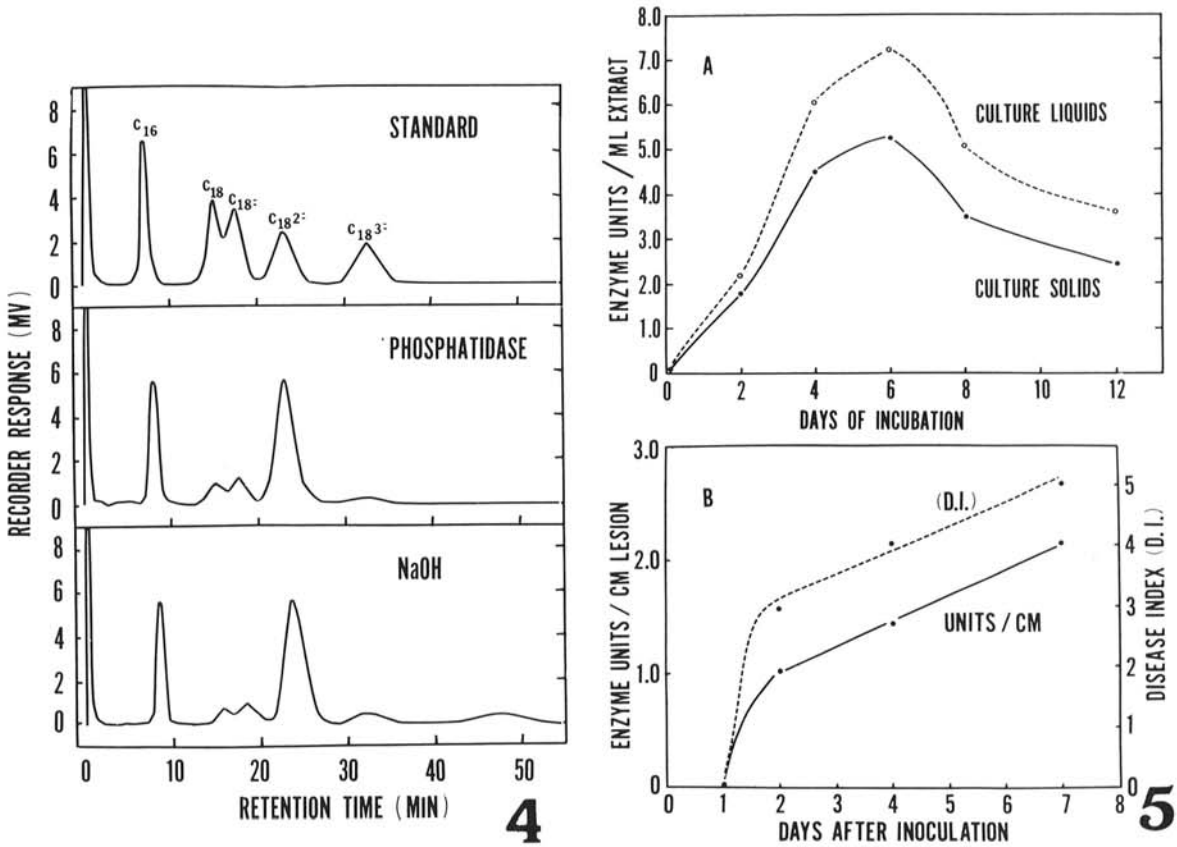

Fig. 4-5. **4)** Gas-liquid chromatogram of standard palmitic (C_{16}), stearic (C_{18}), oleic ($C_{18}=$), linoleic ($C_{18}2=$), and linolenic ($C_{18}3=$) acids compared with fatty acids contained in a chloroform extract of *Sclerotinia sclerotiorum* phosphatidase reaction mixture incubated 4 hr at 30 C with 0.5% lecithin in 0.05 M succinate-NaOH buffer, and compared with fatty acids obtained from a 24-hr hydrolysate of soybean lecithin saponified with 0.6 N NaOH. **5) A)** Phosphatidase activity of extracts of culture solids of *Sclerotinia sclerotiorum* grown on autoclaved bean hypocotyls, and the free liquids from these same cultures. Enzyme reaction mixtures contained 0.5% lecithin in 0.05 M succinate buffer (pH 4.0) and 0.004 M $CaCl_2$ in 1.0 ml total volume. Activity is expressed as µg phosphorus released/ml of reaction mixture. **B)** Phosphatidase activity in slurries prepared from diseased bean hypocotyls harvested 1, 2, 4, and 7 days after inoculation. Activity is expressed as enzyme units (1 unit = enzyme required to release 1 µg of acid-soluble phosphorus/min at 30 C from the above reaction mixture) per cm of hypocotyl lesion. D.I. = Disease index; 0 = healthy; 5 = complete invasion and collapse.

lation, to pH 4.5, 2 days after inoculation, then again approached the pH of healthy tissue (pH 6) 7 days after inoculation.

Several isolates of *S. sclerotiorum* from several locations were tested for their ability to produce phosphatidase in cultures on autoclaved bean hypocotyls. All of the isolates tested produced phosphatidase, but there was no correlation between the amount of phosphatidase produced and the relative ability of each isolate to cause disease of bean under nearly standard conditions (Table 1). For example, isolate Ss-3 produced moderate amounts of the enzyme and was virulent, whereas isolate Ss-1 was nearly avirulent but produced approximately twice as much phosphatidase as Ss-3. A disease index of 1.0 or less indicated very little infection.

DISCUSSION.—*Sclerotinia sclerotiorum* produces extracellular, inductive phosphatidase abundantly on autoclaved bean hypocotyls. A previous report (11) stated that this fungus (*S. libertiana*) produces phospholipase A, B, and C; however, I could find no evidence for the production of phosphatidase C by the Ss-3 isolate. Phosphorylcholine, a product of hydrolysis of lecithin by phosphatidase C, was not detected in the present study. Whether the A and B enzymes are produced and are operating together to account for the hydrolysis of both fatty acid acyl-ester linkages and the release of glycerylphosphorylcholine, or whether the B enzyme hydrolyzes both linkages, will have to be resolved. *Sclerotium rolfsii,* a pathogen similar in many respects to *S. sclerotiorum,* appears to produce only the B enzyme (16). At any rate, *S. sclerotiorum* produces a phosphatidase(s) that catalyzes the release of both of the fatty acids from soybean lecithin, releasing acid-soluble glycerylphosphorylcholine. Calcium chloride stimulates the catalysis, and the optimum pH for activity is at pH 4.0. The optimum pH for enzyme activity and stimulation by $CaCl_2$ are reasonably consistent with previous reports (10, 11).

Although enzyme production is abundant in the nat-

ural medium, apparently *S. sclerotiorum* is not able to produce phosphatidase in appreciable amounts in a synthetic medium with soybean lecithin as a carbon source. Also, soybean lecithin does not appear to support abundant growth of the fungus, nor does it act as an inducer of phosphatidase even when glucose is provided as the source of carbon. Whether phosphatidase plays a role in the nutrition of *S. sclerotiorum* remains to be determined.

The present study and a preliminary report (6) are apparently the first to associate *S. sclerotiorum* phosphatidase with diseased plant tissue. The fact that phosphatidase is produced early in pathogenesis, and that it is correlated with symptom expression, suggests a causative effect in disease development. The sharp drop in the pH of diseased tissue extracts shortly after inoculation would enhance activity of this enzyme with its pH optimum for activity at pH 4. There appears to be no correlation, however, between the relative virulence of *S. sclerotiorum* isolates from various sources on bean (Table 2) and the ability to produce large quantities of the enzyme in culture. This is not surprising, as there are undoubtedly many criteria determining pathogenesis. It is significant, however, that all the isolates tested are capable of producing phosphatidase, and all the isolates caused some degree of infection. The role phosphatidase may play in diseases caused by *S. sclerotiorum* is being investigated.

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