Isolation and Partial Characterization of an Acid Endoprotease Present in Dormant Apple Shoot Bark

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ABSTRACT

A major protease present in dormant bark tissues of the apple (Malus domestica Borkh. cv. “Golden Delicious”) was partially purified by hemoglobin-coupled Sepharose column chromatography. The protease active at pH 4.6 and at temperatures of 30 to 50°C was found to be sulfhydryl-dependent. Phenylmercuric acetate inhibited the enzyme approximately 50% at 2 millimolar, whereas phenylmethylsulfonyl fluoride inhibited less than 30% even at 10 millimolar. Substrate specificity and the separation of the enzyme reaction products indicated that the enzyme is likely to be an endoprotease. We suggest that the storage proteins in apple bark tissue undergo some modification prior to their eventual hydrolysis to amino acids, which requires a multi-enzyme system(s). Evidence is presented that the apple bark protein extracts enzymically release ninhydrin-positive compounds upon storage at 5°C. It is concluded that the activation of the sulfhydryl-dependent acid endoprotease is associated with the rapid metabolism of storage proteins which accompanies bud break upon regrowth.

It has recently been recognized that proteins in apple shoot bark are of prime importance in supplying nitrogen for early spring growth (17, 25, 26). However, the enzymic breakdown of these proteins in the apple has not been documented. Tromp and Oava (25, 26) reported the spring mobilization of protein nitrogen in apple shoots, but did not study the enzymes involved. To understand the biochemical sequence of events involved in the annual cycling of nitrogenous compounds in the apple, we have reported the enzymic steps of nitrogen mobilization and conservation during autumnal senescence (11, 12). Here, we report the separation and a partial characterization of a sulfhydryl-dependent acid endoprotease present in dormant apple shoot bark.

MATERIALS AND METHODS

Plant Materials. One-year-old shoots were collected during the winter period of 1978 to 1979 from 6-year-old apple trees (Malus domestica Borkh. cv. “Golden Delicious” on M 26 rootstocks) grown in the orchard of the Department of Horticulture. All studies were conducted with the bark of the shoots. The shoot samples were used immediately after collection or stored at −20°C until used.

Preparation of the Affinity Column. The coupling of the denatured Hgb1 to CNBr-activated Sepharose 4B was based on the manufacturer’s recommendations (20), with some modifications. Five g solid Sepharose 4B were extensively washed with 1 mM HCl at room temperature. To the gel suspension was added the protein solution (0.2 g denatured Hgb dissolved in 20 ml of 0.1 M NaHCO3 containing 0.5 M NaCl, pH 8.3). The mixture was allowed to stand overnight at 5°C with occasional stirring. The Hgb solution was then decanted. The residual active groups were blocked by suspending the gel in 200 ml of 1 M ethanolamine (pH 9.0), for 3 h at room temperature. The remaining uncoupled Hgb and the blocking agent were washed away with 0.1 M NaHCO3 in a sintered glass funnel (medium porosity) with mild suction. The gel suspension was then washed alternately five times with 200 ml each 0.1 M NaHCO3 (pH 8.3), and 0.1 M sodium acetate-acetic acid buffer (pH 4.0), both containing 0.5 M NaCl. After the final wash with the acetate buffer, the gel was thoroughly washed with 50 mM sodium phosphate buffer (pH 6.0). The gel was then packed into a column (1×14 cm) and washed until the wash showed no A at 280 nm. The column was carefully washed prior to each use.

Enzyme Isolation. The bark was separated from the shoot wood, cut up into small pieces with scissors, and weighed immediately. Twenty g bark were routinely used for enzyme extraction. The bark was extracted with 0.1 M sodium phosphate-citric acid buffer (pH 6.0), containing 0.4 M NaCl and 6 mM cysteine, using a 2:1 ratio of insoluble PVP to tissue. The PVP was hydrated overnight in the extraction medium prior to use. Extractions were carried out twice using a Brinkmann Polytron at a setting of 7 for 30 s until tissue disruption appeared to be complete. The homogenate was filtered through eight layers of cheesecloth and centrifuged at 17,000g for 30 min.

The supernatant was brought to 40% saturation with solid ammonium sulfate. The resultant suspension was allowed to stand for 1 to 2 h at 0°C. It was then centrifuged at 22,000g for 30 min. Preliminary experiments showed that less than 5% of the total protease activity was present in the pellet. The supernatant was brought to 75% saturation with solid ammonium sulfate, and the suspension was allowed to stand for 3 to 5 h at 0°C. The 40 to 75% fraction was resuspended in 7 ml of 50 mM sodium phosphate, (pH 6.0), and passed through a column of Sephacryl S-200 Superfine (2.5×60 cm) previously equilibrated with 25 mM sodium phosphate buffer (pH 6.0). The high mol wt effluent (55–65 ml) containing most of the protease activity was layered on the affinity column previously equilibrated with the same buffer as above. The bulk of the other proteins was eluted with the equilibrating buffer. When the A at 280 nm of the eluate had declined to a constant level, the reservoir buffer was replaced with 0.1 M acetic acid (pH 3.1) to release the proteases. The pH 3.1 eluate was quickly neutralized with 4 M sodium acetate and pooled.

Preparation of Substrates. Unless otherwise specified, 2 g denatured Hgb (Sigma) were dissolved in 100 ml water or 0.1 M sodium acetate buffer (pH 4.6), wherever appropriate. Azolebumin (Sigma), RuBP carboxylase of Spinach (Sigma), casein (Miles Research), and BSA (General Biochem, fraction V) were dissolved

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1 This research was partially funded by the Illinois Agricultural Experiment Station. This report is taken in part from the Ph.D. dissertation submitted by S. M. Kang to the Graduate College of the University of Illinois at Urbana-Champaign.

2 Abbreviations: Hgb, hemoglobin; RuBP, ribulose 1,5-bisphosphate; Leu-NA, leucine nitrilamide; Z-phe-alA, carbobenzoxycoprolyl-ala-nine; PMSF, phenylmethylsulfonyl fluoride; PMA, phenylmercuric acetate; Hgb-ase, hemoglobin-hydrolyzing enzyme; ABP-ase, apple bark protein-hydrolyzing enzyme.
in water to make a 2% solution and simply substituted for Hgb in
the standard assays.
Leu-NA (28.8 mg) was dissolved in 1 ml dimethyl sulfoxide (3)
diluted to 50 ml with 25 mM sodium phosphate buffer, (pH 7.0). The final concentration was 2 mM. One ml of this solution
was added to 3 ml reaction mixtures. Z-phe-al a was dissolved in
0.1 M potassium acetate buffer (pH 4.6) to a final concentration of
2 mM (27).
The apple bark protein was extracted as described above. To
the supernatant was added concentrated NaOH for a final
concentration of 2 M. The mixture was allowed to stand at room
temperature for 2 to 3 h and then centrifuged at 17,000g for 30
min. Concentrated trichloroacetic acid solution was added to the
supernatant to give a final concentration of 20%. After centrifuga-
tion at 12,000g for 20 min, the protein pellet was resuspended
in a small volume of 0.1 M NaOH, containing 8 M urea, and
allowed to stand overnight at room temperature. The protein
solution was then extensively dialyzed against water. The final
concentration was adjusted to 10 mg/ml. No increase in the
ninhydrin-positive compounds was observed when this protein
solution alone was incubated for 3 h at 40°C.
Enzyme assays. Unless otherwise indicated, the standard reaction
mixtures contained: 0.5 ml of 0.1 M sodium acetate buffer
(pH 4.6), 0.2 ml of 2% Hgb dissolved in the assay buffer, and 0.2
ml enzyme preparation. Reactions were run at 40°C for 3 h and
terminated by adding 0.1 ml of 50% trichloroacetic acid. Incuba-
tion mixtures containing only substrate or enzyme solution, or
boiled enzyme (100°C for 5 min) in the same assay buffer, were
similarly treated and analyzed to serve as controls. The protein
precipitate was removed immediately by centrifugation in a Beck-
man Microfuge B for 3 min, and the ninhydrin-positive com-
pounds were determined by the method of Moore and Stein (16).
To ensure measurement of protease activity under optimum con-
ditions, assays were also conducted over different time intervals
and with varying amounts of enzyme added to the same standard
reaction mixtures.
Unless otherwise specified, protease activity is expressed as µg
α-NH₂ released per h under the standard assay conditions as
described above.
Inhibitor Studies. Appropriate amounts of PMSF and PMA
were dissolved in 100% ethanol, keeping the final concentra-
tion of ethanol at 4.4%. Controls at this level of ethanol without
the inhibitors showed no effect on protease activity. EDTA, 1,10-
phenanthroline, and β-mercaptoethanol were dissolved in the
assay buffer. The inhibitors were added to the enzyme solutions,
and the mixtures were preincubated at 25°C for 30 min prior to
the standard assays. Control preincubation mixtures contained
equal amounts of the corresponding solvents.
Breakdown of Apple Bark Proteins in Solution. The crude
protein extracts were stored at 5°C for 10 days, with or without
prior boiling at 100°C for 5 min. Another batch of crude extracts
was stored in the presence of 2 mM PMA added as an ethanolic
solution. Changes in ninhydrin-positive compounds were meas-
ured after the indicated period of storage.
Immediately after extraction, an aliquot of the extracts was
passed through a Sephacryl S-200 Superfine column (2.5 × 60 cm)
equilibrated with 25 mM sodium phosphate buffer (pH 6.0). High
molecular weight proteins were easily separated from the ninhydrin-positive compounds. The proteins containing protease activity were
stored at 5°C for 5 days and then rechromatographed on the same column
under the same conditions. The reappearance of ninhydrin-positive
compounds was taken to indicate the enzymic breakdown of
apple bark proteins in these solutions.
Protein Determination. Two ml of crude extracts were mixed
with 2 ml of 2 M NaOH, and allowed to stand for 1 to 2 h at room
temperature. The insoluble material was removed by centrifuga-
tion. The NaOH solubilization step was omitted with the protein
solutions containing little or no chromophores after the column
chromatography. Three ml 50% trichloroacetic acid (w/v) were
added to the supernatant. The protein pellet was resuspended in
an appropriate volume of 0.1 M NaOH. Protein was then measured
by the method of Lowry et al. (14) and/or by the dye-binding
method of Bradford (1), using BSA as a standard.
The method of dye binding yielded significantly lower values
than did the Lowry procedure. However, a consistent quantitative
relationship existed between the values obtained by the two pro-
cedures. Therefore, the apparent protein content as estimated by
the dye-binding method was readily converted to equivalent val-
ues for the Lowry method.
RESULTS AND DISCUSSION
Enzyme Purification. The apple bark proteases were precipi-
tated with 40 to 75% ammonium sulfate and partially purified
by gel filtration and affinity chromatography (Table I). The loss of
Hgb-ase activity occurred mainly in the ammonium sulfate frac-
tionation step, which involved a 30% loss of proteolytic activity.
After gel filtration, little loss of proteolytic activity was observed
during the affinity column chromatography. A 40-fold purification
was easily achieved with 65 to 70% recovery of the Hgb-ase
activity. With the crude extracts, it was found that caution was
necessary to measure the proteolytic activity because the back-
ground was high in ninhydrin assays, and the amount of ninhy-
 drin-positive compounds gradually increased upon incubation
with no substrate added.
Figure 1 shows the elution profile of partially purified apple
bark proteases on the affinity column. More than 90% of the
protein was washed through the column with the equilibrating
buffer at pH 6.0. The initial wash is termed the "void protein
fraction". The proteases adsorbed onto the column were eluted by
changing the elution buffer to 0.1 M acetic acid (pH 3.1). Less than
7% of the protein applied to the affinity column was collected in
the protease fractions. The specific activity was constant among
the fractions which contained Hgb-ase activity. Rapid neutrali-
zation was necessary to stabilize the enzymes. Disc gel electropho-
retic separation of the protease-active proteins revealed at least six
different protein species.
A small amount of Hgb-ase activity was observed in the void
protein fractions (Fig. 1). This may be considered as proteases
which have little or no biological activity with Hgb. No attempt
was made to isolate the protease(s) detected in the void protein
fractions. A similar phenomenon has been reported with germi-
nated barley (2), oat leaves (4), and wheat leaves (8). Burger (2)
reported that the amount of proteolytic activity detected in the
void protease fraction was proportional to the amount of enzyme
applied to the column.
Characteristics of the Hgb-ase. The Hgb-ase activity was di-
rectly proportional to the amount of enzyme added up to 100 µg
protein in the standard reaction mixtures (data not shown). The
enzyme exhibited a high temperature optimum of 30 to 50°C (data

| Table 1. Purification of Proteases Extracted from Dormant Apple Shoot Bark |
|-----------------|----|-----|-----------------|-----------------|
| Fraction        | Volume | Protein | Total Activity | Specific Activity |
|                  | ml    | mg/ml | units* | units/mg protein |
| Crude extract   | 283   | 1.1   | 338   | 1.1             |
| 40-75% (NH₄)₂SO₄| 62    | 1.2   | 243   | 3.3             |
| Affinity chromotography | 28 | 0.2 | 222 | 39.6          |

* One unit of enzyme activity is defined as 1 µg α-NH₂ released/h under
the standard assay conditions.
greater

than

the

sured

by

Maximal

extracts.

endosperm

(27).

differential

sensitivity

changed

KANG

proteases

present

(Fig.

5.6)

been

proteins

were

shown

proteolytic

activity

was
determined

at

pH

4.6

under

the

standard

Hgb

assays

by

measuring

ninhydrin-positive

compounds

(——). The changes

in

pH

of

the

fractions

are

shown

(— △—).

not

shown). In this temperature range, the rate of proteolysis was

linear with time for 180 min. At 20 °C, the enzyme exhibited only

30% of the rate of proteolysis as at 40 °C. High temperature optima

of

acid

Hgb-ase

have

been

reported

in

oat

leaves

(4),

germinated

sorghum

(9),

and

maize

endosperm

(10). It seems that a high

temperature optimum is characteristic of the acid proteases. This

may indicate that the protease protein is more stable than other

enzyme

proteins

in

senescing

leaves

and

apple

bark.

The

presence

of

the

substrate

appears

to

be

necessary

to

stabilize

the

enzyme

at

temperatures

above

30 °C. Incubation of the enzyme

at

temperatures

above

30 °C for 1 h in the absence of the

substrate

resulted

in

a

significant

enzyme

inactivation

(data

not

shown).

Garg

and

Virupaksha

(9)

and

Peoples

and

Dalling

(18)

reported

inactivation

of

acid

proteases

at

temperatures

above

25 to 30 °C

for

10 min in the absence

of

the

substrate.

pH Optimum. The apple bark Hgb-ase

was found
to be active

over a pH range of 4.2 to 4.8, with a distinct optimum at pH 4.6

(Fig. 2). The activity sharply decreased above pH 5.0, with no

significant

Hgb-ase

activity

at

pH

values

over

7.0. The pH

opti-
mum

of

the

apple

bark

protease

is

similar
to

that

of

the

Hgb-ases

from

senescing

oat

leaves

(4),

wheat

leaves

(7),

and
castor

bean

endosperm

(27).

The

proteolytic activity against denatured apple bark proteins

(ABP-ase)

showed

maximal

activity

at

pH 5.3, with a relatively

broad

range

of

pH

from

4.6 to 5.4 (Fig. 2). The same pH profile

was observed with the proteins denatured by boiling the crude

extracts. Maximal proteolytic activity against native proteins has

previously

been

shown

to

be

around

pH 5.0 (18, 19, 24).

In

the

pH

profiles,

two

shoulders

with

Hgb-ase

(pH values 4.0

and

5.6) and one shoulder with ABP-ase (pH 3.6) appeared to be

present

(Fig. 2). Similar results have been reported with the acid

proteases

of

oat

leaves

(4) and wheat (18). The shoulder(s) and, especially with the ABP-ase, a relatively broad pH optimum below

pH 5.6 may suggest the presence of more than one enzyme.

Substrate Specificity. The rate of proteolysis of Hgb, as

measured

by

the

release

of

ninhydrin-positive compounds, was always

greater

than

that

of

the

denatured
dark

proteins

(Fig. 2). The
differential

sensitivity

of

the

apple

bark

protease
to

different

substrates

is

further

shown

in

Table II. Casein, BSA, and azoal-
bumin were poorly attacked by the apple bark protease, whereas

the

rate

of

hydrolysis

of

RubP carboxylase

and

denatured

apple

bark

proteins

was

one-third

that

of

Hgb. However, it is possible

that

the

ABP-ase

was

not

measured at its optimum conditions.

This

remains
to

be

clarified.

The

differential

sensitivity

of

nonphysiological

substrates

has


Table II. Hydrolysis of Various Proteins by the Protease Extracted from

Dormant Apple Shoot Bark

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hab</td>
<td>100</td>
</tr>
<tr>
<td>Casein</td>
<td>4</td>
</tr>
<tr>
<td>BSA</td>
<td>10</td>
</tr>
<tr>
<td>Azoalbumin</td>
<td>5</td>
</tr>
<tr>
<td>RuBP carboxylase (spinach)</td>
<td>36</td>
</tr>
<tr>
<td>Apple bark proteins</td>
<td>33</td>
</tr>
</tbody>
</table>

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Plant percent of the of measured under the standard assay conditions, but at the indicated concentrations of Hgb. Reaction velocity is expressed as μg α-NH$_2$ released/h. Inset shows the double reciprocal plot of the saturation curve.

Table III. Effect of Various Compounds on the Activity of the Apple Bark Protease

<table>
<thead>
<tr>
<th>Compound</th>
<th>Residual Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>Na$_2$-EDTA (10)</td>
<td>100</td>
</tr>
<tr>
<td>1,10-Phenanthroline (5)</td>
<td>100</td>
</tr>
<tr>
<td>PMSF (10)</td>
<td>72</td>
</tr>
<tr>
<td>PMA (2)</td>
<td>55</td>
</tr>
<tr>
<td>β-Mercaptoethanol (5)</td>
<td>138</td>
</tr>
</tbody>
</table>

been reported (18, 23, 24). If proteases possess protein specificity, as has been substantiated in some cases (21, 28), the choice of substrate may be critical for the detection of proteolytic activity in vitro. The best substrate might be a protein(s) which disappears (or loses activity) in the tissue during the time period in question. Reports on little correlation between protein depletion and proteolytic activity support this notion.

Kinetics. Figure 3 shows the saturation curve of the Hgb-ase activity of apple bark extracts and includes a double reciprocal plot. The approximate $K_m$ value was 0.088% Hgb. Assuming the mol wt of Hgb is 68,000, 0.088% corresponds to 12.9 μM. The $K_m$ for Hgb of the apple bark protease is lower than that of oat leaf acid protease, 0.115% (5), but higher than that of the acid protease of wheat seedling, 0.026% (6). The $K_m$ for N,N-dimethyl Hgb of the acid protease from germinated barley was 0.064 to 0.067% (2). Although the pH profile (Fig. 2) indicated that the enzyme system could be heterogenous, the double reciprocal plot shows a linear relationship between substrate concentration and the reaction velocity. This suggests that any subspecies would have the same $K_m$. A study which is underway to clarify the number of enzymes involved in this system may solve this problem.

Evidence for an Endoprotease. An attempt was made to elucidate the action mechanism of the acid protease by employing Leu-NA and Z-phe-ala as substrates for aminopeptidase and carboxypeptidase, respectively. The reaction rates of the apple bark protease against these synthetic substrates were less than 10% of those against Hgb (data not shown). It is thus conceivable that the acid protease is an endoprotease. This conclusion is further supported by separating the enzymic reaction products against Hgb in a Sephacryl S-200 Superfine column. Heterogenous sizes of nitrogenous compounds were produced by this enzyme reaction (data not shown). Therefore, it may be concluded that the hydrolysis of apple bark storage proteins to amino acids requires a multi-enzyme system(s). Once a substantial quantity of new termini has been generated by this major endoprotease, exoprotease(s) may then become more important for the complete breakdown of storage proteins.

Effect of Metal Chelators and Inhibitors. The metal chelators EDTA and 1,10-phenanthroline did not affect the enzyme activity at 10 and 5 mM, respectively (Table III). Ten mM phenanthroline caused a 5% inhibition of the enzyme activity. It is thus unlikely that the apple bark protease is a metalloprotease. The small inhibition may be attributable to an ionic strength effect. Aggregation of proteins is often dependent on ionic strength (14), and differences in enzyme activity might thus arise.

PMSF inhibited the apple bark protease activity by 28% in the range from 5 to 10 mM. It is unlikely that the enzyme is a serine-type. The concentration needed to cause less than 30% inhibition appeared to be higher than would be expected. It was not possible to obtain 50% inhibition within the limited range of solubility of this compound. Furthermore, there is evidence that PMSF is not as specific for serine protease as is sometimes supposed (5, 22, 29). Instead, preincubation of the enzyme with PMA resulted in a 45% inhibition of enzyme activity at 2 mM, indicating that free sulphydryl groups participate in catalysis. The stimulation of the enzyme activity in the presence of β-mercaptoethanol could support this conclusion.

Since there appeared to be more than one enzyme present, this would account for the partial inhibition observed in the presence of 2 mM PMA. Until more is known about the mechanism of action of this protease, it seems premature to describe the inhibitor pattern for this enzyme as typical of any one class of protease (22). However, the observation that the enzyme is at least partially inhibited by PMSF indicates that it may be a serine-type endoprotease.
of either PMSF or PMA. (The pH curve shows heterogeneity; it might be possible to detect differences in inhibitor sensitivity by repeating the experiment at different values.) The presence of substrate proteins during enzyme preparation, as well as conformational changes induced by substrate binding, could alter the accessibility of liable groups. The limited solubility of the mercu- rial compound in water could also limit its approach to the relatively hydrophilic centers of the proteins.

**Breakdown of Apple Bark Proteins in Solution.** Crude apple bark protein extracts containing active proteases produced increasing amounts of ninhydrin-positive compounds during storage at 5°C (Fig. 4). This increase in ninhydrin-positive compounds was found to be enzymic since it did not occur in boiled extracts. In nonboiled crude extracts in which proteases are in contact with their substrate proteins, a nearly linear increase was observed for 6 days, resulting in an approximately 60% increase in ninhydrin-positive compounds. The rate of increase leveled off after 2 weeks of storage. However, the addition of 2 mM PMA reduced the rate of increase by one-half. A 30% increase in ninhydrin-positive compounds was observed after 6 days of storage with no significant changes thereafter.

Further evidence for the enzymic breakdown of apple bark proteins in solution is presented in Figure 5. An aliquot of crude extract was chromatographed on a Sephacryl S-200 Superfine column. The Hgb-ase active protein and the fractions containing ninhydrin-positive compounds were separated (Fig 5A). The latter fractions were discarded. Only the protease-active protein fractions were pooled and stored for 5 days at 5°C. They were then rechromatographed on the same column (Fig. 5B). The protein content decreased nearly 35% during this storage period, with a concomitant decrease in proteolytic activity by about 40%. Ninhydrin-positive compounds reappeared after storage, the increase amounting to about 30% over the level at day zero. Since the total protein content apparently decreased in the crude extracts, and since the boiled extract did not exhibit an increase in ninhydrin-positive compounds, we concluded that the ninhydrin-positive compounds which increased upon storage were small peptides and amino acids enzymically generated from the proteins. These results indicate that the proteases degrade their native substrates in solution. It is also possible that the proteases may have undergone autolysis. Martin and Thimann (15) suggested that the rapid decrease in protease activity of oat leaves toward the end of senescence period resulted from self-digestion of the proteolytic enzymes. Drivdahl and Thimann (5) also presented evidence for in vitro autolysis of oat leaf proteases. If the autolytic breakdown of proteases occurred in the enzyme preparation, minor species of proteases could have been generated, thus affecting the pH dependence of the enzyme activity as previously discussed.

In addition to the problem of protease aggregation with other proteins, the enzymic breakdown of apple bark proteins in solution and the possible autolysis of proteases pose an important problem in protein purification and enzyme isolation. The entire purification procedure should be carried out as quickly as possible. In addition to handling extracts at low temperatures, the addition of protease inhibitors such as PMA to the extraction medium could prove helpful.

**Acknowledgments.** The authors are grateful to Professor David B. Dickinson for his valuable discussions and suggestions during the course of this study and manuscript preparation.

**LITERATURE CITED**

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