Enzymatic Properties, Substrate Specificities and pH–Activity Profiles of Two Kiwifruit Proteases

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Summary Kiwifruit (Actinidia chinensis) contains abundant protease, actinidin, and two possible components which were named A1 and A2. However, a comparison of the two components has not been thoroughly conducted. We have previously shown the presence of six proteases named KP1, KP2, KP3, KP4, KP5 and KP6 in kiwifruit, and that each purified kiwifruit protease was chromatographically pure. It was also indicated that the two representative components, KP4 and KP6, must be A1 and A2. To establish whether or not the two proteases, KP4 and KP6, have the same specificity in proteolytic activity, their enzymatic properties were compared. Between the two proteases, differences in substrate specificity against several protein-substrates (casein, gelatin, collagen, ovalbumin and bovine serum albumin) were not observed by digestion-product analysis with sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The kinetic parameters of KP4 against N-\(\varepsilon\)-carbobenzoxy-lysine p-nitrophenyl esters were different from those of KP6. The pH–activity profiles of KP4 and KP6 against S-3-trimethylaminopropyl-lysozyme, a wide-pH range soluble substrate, and N-\(\varepsilon\)-carbobenzoxy-lysine p-nitrophenyl esters were different.

Key Words kiwifruit protease, actinidin, substrate specificity, kinetic parameter, pH–activity profile

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Abbreviations: PAGE, polyacrylamide gel electrophoresis; OVA, ovalbumin; BSA, bovine serum albumin; Z-Lys ONp, N-\(\varepsilon\)-benzoyloxycarbonyl-lysine p-nitrophenylester; E-64, L-3-carboxy-2,3-trans-epoxypropionyl-leucylamide(4-guanidino)butane; DTT, dithiothreitol; TMA-lysozyme, S-3-trimethylaminopropyl-lysozyme; Km, Michaelis constant; V, maximum velocity; kcat, catalytic center activity.
Kiwifruit is native to China and has been developed commercially in New Zealand. Since commercial planting has rapidly expanded in the Northern hemisphere, kiwifruit is now harvested throughout the year. Kiwifruit contains abundant protease, which was first characterized by Arcus (1). This protease was named actinidin [EC 3.4.22.14]. The degree of utilization of kiwifruit protease, however, is not very high. Some properties of actinidin have been studied by several authors (2-10). Although McDowall, using PAGE, suggested that actinidin contains two components (2), the patterns of the two components of the PAGE and their separation by chromatography were not shown. No other comparison of the two components of actinidin have been carried out. Nothing is known about the physiological function of kiwifruit protease which accumulates to very high levels during early fruit development.

We have shown by improved PAGE that there are six proteases in kiwifruit at pH 4.0 (11). Our chromatographic method made it possible to separate the kiwifruit proteases into six components. Analyses of the six components indicated that KP4 and KP6 must be A1 and A2. Multiple forms of cystein proteases were reported in cases of ginger protease (12), bromelain [EC 3.4.22.4] (13) and asclepain [EC 3.4.22.7] (14,15), and comparisons of each component have been conducted.

We studied the enzymatic properties of the two representative proteases KP4 and KP6. The substrate specificities against casein, OVA, BSA, collagen and gelatin were investigated. The kinetic parameters of the two proteases which were standardized by active-site titration were determined against Z-Lys ONp. The pH-activity profiles of KP4 and KP6 against TMA-lysozyme and Z-Lys ONp were assayed. The reported findings may be useful for elucidation of the biochemical role of each kiwifruit protease and applicable in future research on food processing of purified or crude kiwifruit protease.

MATERIALS AND METHODS

Materials. Kiwifruit was obtained from a local fruit market. Hammarsten casein was obtained from Merck (Darmstadt, Germany). Collagen (pepsin-solubilized collagen, type I) was obtained from Koken (Tokyo, Japan). E-64 was obtained from Peptide Institute (Osaka, Japan). Egg white lysozyme was obtained from Tokyo Kasei (Tokyo, Japan). Z-Lys ONp was obtained from Sigma (St. Louis, MO, USA). OVA, BSA and the other chemicals were obtained from Nacalai Tesque (Kyoto, Japan).

Preparation and purification of kiwifruit proteases. Two kiwifruit proteases, KP4 and KP6, were purified by the ion-exchange chromatographic method (11). The crude kiwifruit protease was modified with p-chloromercuribenzoate to get mercuribenzoate-protease which did not autodigest during purification and fractionation, and could be re-activated with some reducing reagents such as DTT. The homogeneity of these kiwifruit proteases was checked using re-chromatography and PAGE, and the purified proteases were used in further experiments. Protein
concentrations were calculated from absorbance at 280 nm on the basis of
$A_{0.1\%,280} = 2.121 \text{ g}^{-1} \text{ cm}^{-1}$ (3) and the molecular mass, $Mr = 23,500$ (11).

**Substrate specificities of KP4 and KP6 against protein substrates.** The sub-
strate specificities of KP4 and KP6 against protein substrates were estimated by
monitoring the SDS-PAGE patterns of the digests. Casein, OVA, BSA, collagen
(pepsin-solubilized collagen, type I) and gelatin were used as substrates. Gelatin
was prepared from collagen by heating at 50°C for 10 min. The reaction was started
by the addition of protease solution, 10 μL (1 μg) of KP4 or KP6 and 10 μL of
1 mM DTT, to the substrates. For casein, OVA or BSA, protease digestion was
performed at 50°C with 1 mL of 1% substrate in 0.1 M phosphate buffer (pH 6.0).
The collagen and gelatin reactions were performed at 25°C in 0.1 M phosphate buffer
(pH 6.0) containing 0.5 M NaCl. Then 10 μL of the reaction mixture was added to
5 μL of 1 mM E-64 in 10% dimethyl sulfoxide to stop digestion by KP4 and KP6 in
the next heating step for SDS-PAGE.

SDS-PAGE was performed according to the method of Laemmli (16). We used
myosin (Mr = 200,000), β-galactosidase (Mr = 116,300), phosphorylase b (Mr =
97,400), BSA (Mr = 66,300), glutamic dehydrogenase (Mr = 55,400), lactate
dehydrogenase (Mr = 36,500), carbonic anhydrase (Mr = 31,000), trypsin inhibitor
(Mr = 21,500), lysozyme (Mr = 14,400) and aprotinin (Mr = 6,000) as the molecular
mass markers (TEFCO, Nagano, Japan). One percent SDS, 20% glycerol and
0.005% bromophenol blue were added to the final protein solution, and the mixture
was heated in a boiling water bath for 2 min. After electrophoresis, the gel was
stained with Quick CBB (Wako, Osaka, Japan).

**Kinetic parameters of KP4 and KP6 against a synthetic substrate.** To determine
the kinetic parameters of KP4 and KP6 against a synthetic substrate, the reaction
rate was measured using Z-Lys ONp as a substrate. Esterase activities were assayed
in 40 mM acetate buffer (adjusted with NaOH) containing 10% acetonitrile at 37°C
after the method of Silverstein (17). Seven substrate concentrations ranging between
13 and 400 μM were used. The release of p-nitrophenol was monitored continuously
at 320 nm on a Beckman DU640 spectrophotometer (Watertown, MA, USA)
equipped with a temperature-controlled cuvette. The initial speed was corrected for
spontaneous hydrolysis of the substrates. Each active protease was quantified by
active-site titration according to the method of Kaneda et al (18), except that
N-α-carbobenzoxy-tyrosine p-nitrophenyl ester was used as a titrant. The kinetic
parameters, $Km$ and $V$, were determined by Lineweaver-Burk plots.

**Effects of pH on the activities of KP4 and KP6.** pH-activity profiles were
carried out using TMA-lysozyme and Z-Lys ONp as substrates. The two kiwifruit
proteases were activated by mixing excess DTT. Protease activities against TMA-
lysozyme were determined by measuring the absorbance of trichloroacetic acid-
soluble peptides at 280 nm after the method of Anson (19). TMA-lysozyme was
prepared according to the method of Okazaki et al (20).

Esterase activity against Z-Lys ONp was assayed as described above, except
that the reaction solution contained 0.35% acetonitrile. The time course of hydrolysis

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for each pH was monitored continuously by the release of p-nitrophenol at 320 nm. The changes in the molecular absorbance of p-nitrophenol at various pHs at 320 nm were corrected.

RESULTS AND DISCUSSION

Substrate specificities of KP4 and KP6 against protein substrates

We have shown that kiwifruit proteases were autodigested during the heating step for SDS-PAGE but could be inactivated with E-64 to prevent autodigestion (11). Therefore, E-64 was used to stop the digestion of substrates.

The representative components, KP4 and KP6, in kiwifruit reacted with casein showing similar SDS-PAGE patterns through 120 min. Therefore, they had similar substrate specificity and specific activity against casein (Fig. 1). Furthermore, KP4 and KP6 had similar substrate specificity against other protein substrates. While the activities of KP4 and KP6 against casein were strong, activity against BSA, OVA and collagen was not observed (Figs. 2 and 3).

Cysteine protease inhibitors were found in serum and egg white (21–23). Therefore, it was presumed that KP4 and KP6 might be inactivated by these inhibitors, which exist as contaminants in BSA and OVA. To confirm whether or not any inhibitor was present in BSA and OVA, the activity was estimated using casein substrate in the presence of an equal quantity of BSA or OVA. Even in these cases, KP4 and KP6 could attack casein coexisting with BSA or OVA (Fig. 2). Therefore, such an inhibitor in BSA or OVA was not present. These results suggest that KP4 and KP6 cannot digest native BSA or OVA, which have disulfide bonds, but can digest natural casein which does not have disulfide bonds.

![Fig. 1. SDS-polyacrylamide gel electrophoresis of casein digested by KP4 and KP6 on 18.0% polyacrylamide gel. The digestion was carried out at 50°C with 1 mL of 1% casein in 0.1 M phosphate buffer (pH 6.0) for 0, 20, 40, 60 and 120 min. The reaction was stopped with E-64.](image-url)
Fig. 2. SDS-polyacrylamide gel electrophoresis of various substrates digested by KP4 and KP6 on 18.0% polyacrylamide gel. The digestion was done at 50°C with 1 mL of 1% substrates in 0.1 M phosphate buffer (pH 6.0) for 0, 10, 20, 30 and 60 min. The reaction was stopped with E-64.

The reaction of kiwifruit protease against gelatin is known (1, 3), and we confirmed it in this study (data not shown). The reaction against collagen was performed at 25°C where no heat-denaturation occurs. Collagen molecules consist of triple helix and globular domains which form crosslinks. The globular domain is referred to telopeptide, which is susceptible to many proteases (24). However, a natural triple helix can be cleaved only by collagenase; collagenase activity is defined as the action against a triple helix domain in a collagen molecule (25, 26). It has been reported that the β-chain of acid-solubilized collagen was converted to an
Fig. 3. SDS-polyacrylamide gel electrophoresis of collagen digested by KP4 and KP6 on 7.5% polyacrylamide gel. The digestion was carried out at 25°C with 1 mL of 1% collagen in 0.1 M phosphate buffer (pH 6.0) containing 0.5 M NaCl. The reaction was stopped with E-64.

Table 1. Kinetic parameters of KP4 and KP6.

<table>
<thead>
<tr>
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<th>$K_m$ (mm)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (mm$^{-1}$·s$^{-1}$)</th>
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<tbody>
<tr>
<td>KP4</td>
<td>0.044 ± 0.001</td>
<td>34.05 ± 1.07</td>
<td>774</td>
</tr>
<tr>
<td>KP6</td>
<td>0.053 ± 0.003</td>
<td>42.96 ± 1.41</td>
<td>811</td>
</tr>
</tbody>
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Hydrolysis of Z-Lys ONp was assayed in 40 mM acetate buffer (pH 5.5, adjusted with NaOH) and 10% acetonitrile at 37°C. The values shown are means ± SD ($n=3$).

$\alpha$-chain by kiwifruit protease (27).* However, those results indicated that kiwifruit protease acted against telopeptide. In this study, using pepsin-solubilized collagen which does not have telopeptide, no significant cleavage of the triple helix domain of collagen was observed (Fig. 3). Therefore, we concluded that KP4 and KP6 have no collagenase activity.

**Kinetic parameters of KP4 and KP6 against a synthetic substrate**

It was thought that KP4 and KP6 contained one single SH group per molecule (8), but KP4 contained 0.70 and KP6 contained 0.59 SH groups per molecule, the protein concentration of which was determined from $A_{0.1\%280}=2.121$ g$^{-1}$ cm$^{-1}$

(3). Their low values of SH groups may reflect some irreversible oxidation of the active SH group site, a common problem in cysteine proteases (14). Initial speeds were monitored over the period from 10 to 130 s. Kinetic parameters were calculated from the initial speeds, which were found to be linear during the first 60 s. The values of kcat/Km in Table 1 show that Z-Lys ONp is an excellent substrate for both kiwifruit proteases. The values of Km, kcat and kcat/Km for KP4 against Z-Lys ONp were different from those for KP6. Similarities of the kinetic parameters were observed for members of groups A and B of asclepains (15).

Effects of pH on the activities of KP4 and KP6

The effects of pH on the activities of KP4 and KP6 against TMA-lysozyme and Z-Lys ONp were investigated. TMA-lysozyme was used as a protein substrate that was soluble in a wide pH range.

The high activity of kiwifruit proteases in low pH regions was explained by the negative-charge groups on kiwifruit protease molecules, because these enzymes have very low pI{s} (pI 3.45 and pI 3.33) (11). However, the findings of this study show that the pH–optimum of each kiwifruit protease against the protein substrate TMA-lysozyme was neutral (Fig. 4). This maintenance of high activity at neutral pH may be due to the high positive-charge of the substrate. Hashinaga et al, using casein as a substrate, reported that crude kiwifruit protease had two pH–optima, pH 3.9 and pH 6.5 (28). Since a casein solution rapidly precipitates near pH 4.5 to 5.0, their results were considered to be due to the isoelectric precipitation of casein (i.e., decrease in substrate concentration). For Z-Lys ONp, kiwifruit proteases maintained high activities at neutral pH (Fig. 5). As for the esterase activity of kiwifruit protease on N2-benzoyl-L-arginine ethyl ester hydrochloride, a broad

Fig. 4. The pH–activity profiles of KP4 and KP6 with S-3-trimethylaminopropyl lysozyme. Protease activity was assayed with 1 mL of 1% TMA-lysozyme in 25 mM acetate buffer at 50°C for 15 min, and stopped with 1.5 mL of trichloroacetic acid. The absorbance of the supernatant at 280 nm by 1 µg protease is shown on the ordinate. ■, KP4; ●, KP6.

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Fig. 5. The pH–activity profiles of KP4 and KP6 with Z-Lys ONp. Esterase activity was assayed with 17.6 μM Z-Lys ONp in 50 mM acetate buffer and 0.35% acetonitrile at 37°C, and was expressed in absorbance at 320 nm per 0.5 μg protease and 5 min. ■, KP4; ○, KP6.

Furthermore, it was shown that pH–activity profiles of KP4 and KP6 were slightly different (Figs. 4 and 5). In pH–activity profiles with TMA-lysozyme, there was a plateau of activity by KP4 in the range from pH 4 to 8, and persistent activities were still observed at pH 3.5 and 9.2 (Fig. 4). The optimum pH range of KP6 was from pH 4.5 to 5.5. KP4 had higher activity than KP6 in the range from pH 5.5 to 8.2. Similar tendencies of KP4 and KP6 were also observed against Z-Lys ONp (Fig. 5). These results indicate that the two proteases have different properties. These findings may be useful information in the processing of kiwifruit proteases.

REFERENCES

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