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RESEARCH NOTE

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The Enzymatic Properties of Actinidine from Kiwifruit

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Abstract Activity and stability of kiwifruit actinidine was determined in various conditions of pH, salt, and temperature using N-α-CBZ-lysine P-nitrophenyl ester as the substrate. Actinidine activity was low below pH 6, and undetectable below pH 3. The enzyme was stable in a pH range of 6.0-8.5. At 4°C the enzyme was inactive in the presence of greater than 36% vinegar and in 2 M NaCl. Actinidine at 25°C was unstable in 24% vinegar but stable in up to 3 M NaCl. With regard to freeze-thaw stability, actinidine retained 85% residual activity after being frozen at -20°C for 3 days. Based on Arrenius and Lineweaver-Burk plots, actinidine became unstable at greater than 45°C with only 30% residual activity remaining after 6 min. The Km, kcat, and kcat/Km values of actinidine were 56 μM, 67/sec, and 1.2 μM/sec, respectively.

Keywords: actinidine, activity, stability, kinetics, thermodynamics

Introduction

Actinidine (EC. 3.4.22.14) is the most abundant protease found in the berries of *Actinidia Chinensis* (kiwifruit). This cysteine protease was first characterized by Arcus (1), and later studied by many other authors (2-5). Actinidine has been shown to have many similarities to papain (from papaya), ficin (from fig), and bromelain (from pineapple) with regard to steady state kinetic behavior, specificity, and physical properties (5-7). These cysteine proteases differ in their amino acid composition, isoelectric points, molecular weights, and carbohydrate content (8-10). For example, the isoelectric point of actinidine is 3.1, which is very different from the values for papain (pH 8.7), ficin (>pH 9), and bromelain (pH 9.5). Actinidine has a molecular weight of 23.5 kDa, compared with 23.4, 25.5, and 33.5 kDa for papain, ficin, and bromelain, respectively. Recently, it was reported that actinidine in kiwifruit exists in three different isoforms measuring 24, 28, and 30 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), immunobloting, and N-terminal sequencing data (7, 8).

Actinidine has a broad optimum pH range of pH 4.0 to 4.3 for 4% gelatin as a substrate, and from pH 5 to 7 using N-α-CBZ-lysine p-nitrophenyl ester (pNPE), N-benzoyl-L-arginine ethyl ester, or N-benzoyl-L-arginine p-nitroanilide as substrates (11-13). Several studies have found that there are three ionizations affecting actinidine activity reflected in pKa values of 2.7-3.1, 4.9-6.5, and 9.7. The pKa values of 2.7-3.1 and 9.7 are assigned to formation of the ion pair (deprotonation of the histidine at high pH and protonation of the cysteine at low pH) and the pKa of 4.9-6.5 is assigned to the catalytical role of enzyme (11, 14, 15).

Actinidine in kiwifruit is known to affect taste or flavor, allergenic properties, and is used as a meat tenderizer. The enzyme prevents gelatin from setting and produces off-flavors when present in milk or milk products for long periods. However, little or no proteolytic effects on milk or gelatin or the setting of pork myofibrillar proteins are observed following brief cooking of the fruit, or the addition of vinegar or salt. Therefore, understanding the properties of this enzyme for practical use would be helpful to prevent unwanted changes or effects in foods treated with actinidine during food processing and storage.

The purpose of this paper is to study the properties of actinidine from commercial kiwifruit extract powder with respect to pH, vinegar, and salt concentrations as well as its kinetics and thermodynamics, thus providing valuable information for the practical use of actinidine as a meat tenderizer or as an ingredient in gelatin based foods and dairy products.

Materials and Methods

Materials Actinidine extract powder was obtained from Natures Food Ingredients Limited (Pukekohe, Australia). Buffer salts and acetonitrile were obtained from Fisher Scientific (Pittsburg, PA, USA), pNPE from Sigma Chemicals (St. Louis, MO, USA), the Bicinchoninic Acid (BCA) protein determination kit was obtained from the Pierce Chemical Co. (Rockford, IL, USA), and filters from Gelman Sciences (Ann Arbor, MI, USA).

Enzyme preparation and assay Actinidine extract powder was dissolved in water and vacuum-filtered through a 1.5 μM filter followed by a 0.2 μM membrane filter to completely remove insoluble particles from the crude enzyme. The protein concentration and profile of this solution was determined using the BCA kit and 10% SDS-PAGE analysis. Actinidine activity (20 μg of actinidine) was monitored by its esterase activity on pNPE (0.13 mM) as described by Boland and Hardman (16). The stock solution of substrate (2.5 mM) was prepared with 90%

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acetonitrile. The hydrolysis of pNPE was measured using a double beam UV-visible spectrophotometer (Biospec 1601; Shimadzu Scientific Instruments, Japan) over 1 min at 400 nm in a 1 mL cuvette. A blank without substrate was used as a control. All experiments were carried out in triplicate at 25°C in 20 mM sodium phosphate buffer, pH 7 unless otherwise stated.

Enzyme activity measurement The optimum pH for actinidine activity was determined by measuring reaction rates over a pH range of 2.0-10.5. The buffers used were: sodium citrate buffer (pH 2-4), sodium acetate (pH 4-5), sodium phosphate buffer (pH 6-7), Tris-HCl buffer (pH 8-10.5), and glycine-HCl (pH 9-10). Actinidine activity was also determined in various salt concentrations (0-5 M NaCl) with 20 mM sodium phosphate buffer, pH 7.

Enzyme stability measurement Actinidine was incubated in 20 mM sodium phosphate buffers with various salt (0-5 M NaCl) or vinegar concentrations (0-48%) for 24 hr at 25 or 4°C, or in 20 mM buffer solution adjusted to various pH levels (pH 2-10.5) for 2 hr at 25°C. Enzyme activity was then measured using 20 mM sodium phosphate buffer, pH 7, containing 0.13 mM pNPE. Freeze-thaw stability was also measured by thawing the enzyme at 25 °C for 30 min after being frozen at -20°C for 3 days. Residual activity was measured using 0.1 M sodium phosphate buffer, pH 7, containing 0.13 mM pNPE.

Kinetic parameters Various concentrations of pNPE substrate (0.0025-0.25 mM) were used to determine kinetic constants. The kinetic parameters Vmax, Km, and kcat for pNPE were determined from Michaelis-Menton and Lineweaver-Burk plots. The reciprocal of V was graphed vs the reciprocal of [S] and the best-fit line was obtained. Values of Vmax and Km were determined using the equation: 1/V=Km/Vmax [S] + 1/ Vmax. The catalytic center activity, kcat was obtained by dividing Vmax by the total active enzyme concentration.

Thermodynamics Enzyme solutions (200 mg in water) were incubated at 25, 45, 55, 65, and 75°C in a water bath for 30 min. Aliquots of enzyme solution (20 mg) at each temperature were removed and the residual enzyme activity was measured using 0.13 mM pNPE every 6 min during the incubation period. The activation energy (Ea) for enzyme inactivation was obtained from two graphs as described by Anthon and Barrett (17). First, the log of the percent residual activity of heat treated enzyme was graphed against the incubation time for each temperature analyzed. The slope of these lines resulted in a rate constant, *k*, for enzyme inactivation. Next, the natural log of the *k* values at each temperature was plotted against the reciprocal of the temperature in degrees Kelvin (K). The slope of the best-fit line gave an estimate of the value -Ea/R where, Ea is the activation energy and R is the universal gas constant (8.314 J /mol K).

Results and Discussion

Enzyme preparation Kiwi extract powder was dissolved in water and 0.2 μM filtered to obtain only the water soluble enzyme fraction since actinidine accounts for about 50% of the soluble proteins in kiwifruit (8). There are two major bands at 24 and 31 kDa on SDS-PAGE analysis, showing the expected molecular weights of actinidine in kiwifruit (Fig. 1). The enzyme solution was stable for 4 weeks at 4°C.

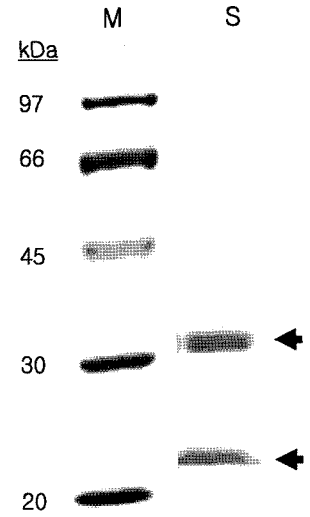

Fig. 1. SDS-PAGE analysis (10%) of actinidine from kiwifruit. Lane M is a protein standard marker. Lane S is fractionated actinidine (10 μg) from crude kiwifruit which was heated after adding SDS and β-mercaptoethanol. Arrows indicate expected molecular weights of actinidine isoforms.

Enzyme activity Enzyme activity was determined under variable conditions of pH (2-10.5) or salt concentration (0-5 M NaCl) (Fig. 2). The specific activity of an enzyme is given as DA/min (or U) per mg enzyme. Actinidine activity was low below pH 6 but increased rapidly at neutral and basic conditions (pH 7-10) as shown in Fig. 2A. This result is consistent with data from Boland and Hardman (16) in which the kcat was pH independent below pH 7.0 but increases greatly at high pH, with a pKa of 8.1. Actinidine was active at 0-0.5 M NaCl but was inactive at 1-5 M NaCl (Fig. 2B).

Enzyme stability Figure 3 shows enzyme stability using 0.1 M sodium phosphate buffers at various pH (pH 2-10.5), vinegar (0-48%) or salt (0-5 M NaCl) concentrations at 25 or 4°C. With regard to pH, actinidine showed the highest stability at pH 7, retaining 100% residual activity (Fig. 3A). Actinidine was stable in the pH range of 6-8.5 with 70% residual activity compared to the activity at pH 7. However, actinidine was almost lost its activity under extreme acidic conditions (below pH 3) (Fig. 3A). Our result is consistent with results of previous studies (11, 18) in that actinidine is unstable outside the pH range of pH 4.3-10.2 and its activity is totally lost at pH 3.2 after 3-5 min incubation (11, 18). The pH dependence of actinidine stability could be due to a combination of the catalytic mechanism at the active-centre (cysteine-histidine ion pair) and electrostatic effects (15, 19). The kinetically significant pKa values of actinidine are 2.7-3.1 (Cys^{25}/His^{162} catalytic site ion pair formation), 4.9-6.7 (Asp^{138}/

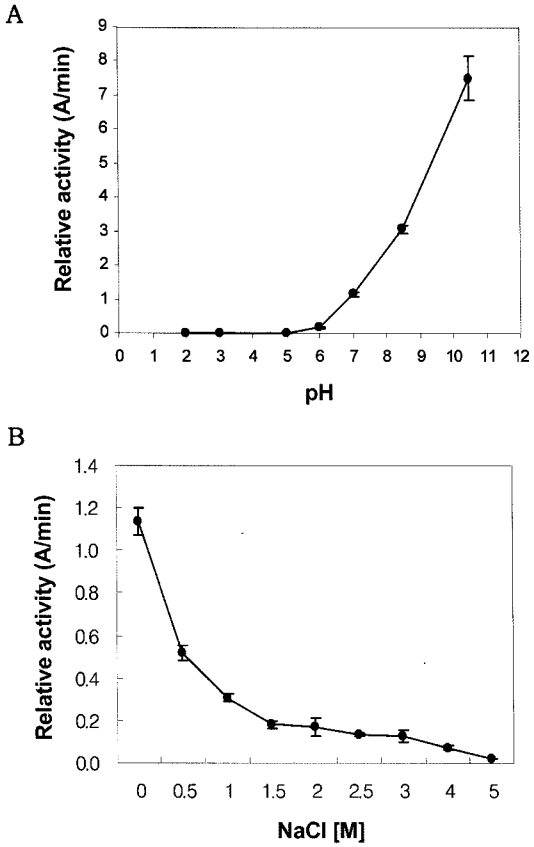

Fig. 2. Actinidine activity with respect to pH and salt concentration at 25°C. A) Effects of pH on actinidine activity (pH 2-10.5), B) Effects of salt on actinidine activity (0-5 M NaCl). Curves represent the mean of triplicates; the standard error is shown by the error bars.

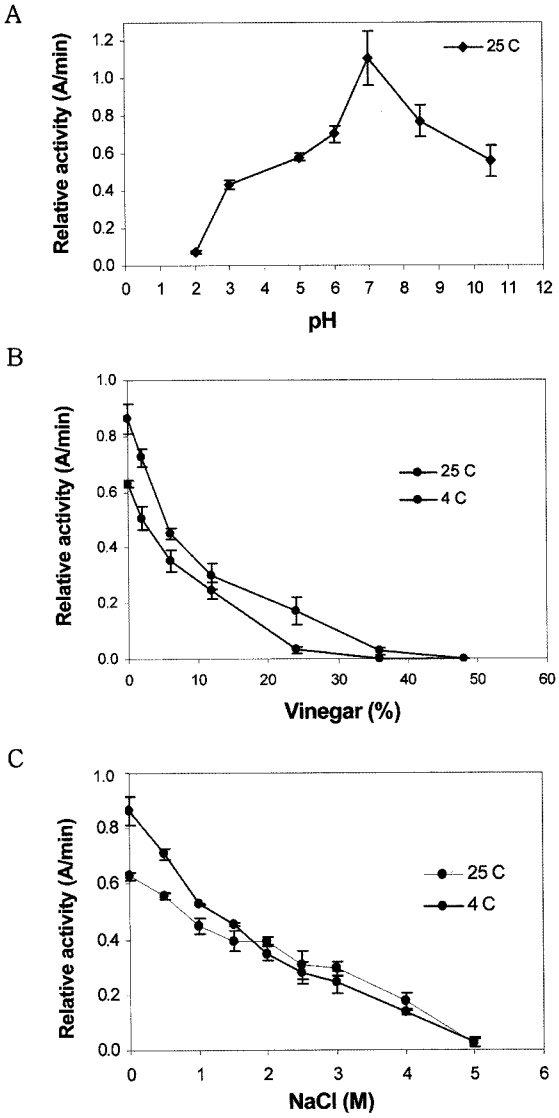

Fig. 3. Actinidine stability with respect to pH, vinegar, and salt concentration at 25 or 4°C. A) Effect of pH on actinidine stability (pH 2-10.5) at 25°C for 2hr, B) Effect of vinegar on actinidine stability (0-48% concentration) at 25 or 4°C for 24 hr, C) Effect of salt on actinidine stability (0-5 M NaCl) at 25 or 4°C for 24 hr. Curves represent the mean of triplicates; the standard error is shown by the error bars.

Asp^{161} couple as electrostatic modulators), and 9.6 (ion-pair deprotonation) (14, 15).

As a practical approach, vinegar (with 5% acidity) was used at concentrations from 0 to 60% in place of buffers (Fig. 3B), and enzyme stability was determined as described earlier. For 24 hr at 4°C, actinidine showed a higher activity than at 25°C at the same vinegar concentration, although similar stability patterns were observed. At 4°C, the enzyme was more stable in 6% vinegar, retaining 58% residual activity in comparison with no vinegar. However, the enzyme became less stable in 24% vinegar with 20% residual activity, and then was virtually lost its activity in greater than 36% vinegar (Fig. 3B). On the other hand, actinidine at 25°C became almost lost its activity in 24% vinegar having less than 5% residual activity (Fig. 3B).

Actinidine activity was stable in salt within a range of 0-5 M NaCl at both 4 and 25°C as shown in Fig. 3C. Enzyme incubated at 4°C showed a higher activity than that at 25°C over a 24 hr period at the same salt concentration, although samples at both 4 and 25°C lost their activities gradually as the salt concentration was increased. Actinidine activity at 4°C was stable at 0-1 M NaCl with more than 60% residual activity, but decreased its activity at 2 M NaCl, showing 40% residual activity. Actinidine activity at 25°C was stable up to 3 M NaCl with 50% residual activity (Fig. 3C).

With regard to freeze-thaw stability, actinidine retained 85% residual activity after being frozen at -20°C for 3 days followed by thawing the enzyme at 25°C for 30 min.

Kinetic parameters A Lineweaver-Burk plot of actinidine hydrolysis of pNPE (0.0025-0.25 mM concentration) is shown in Fig. 4A. The Km, a substrate binding indicator for the enzyme is 56 μM, higher than the 22-28 μM (16, 20) but lower than the 91 μM (12) previously reported for pNPE. The kcat value of 67/sec in this study is higher than 29-33/sec (16, 20) but lower than 101/sec (12) as previously reported. In comparison to other studies, the different enzyme affinity for substrate (Km) and catalytic activity of the enzyme (kcat) in this study may be due to

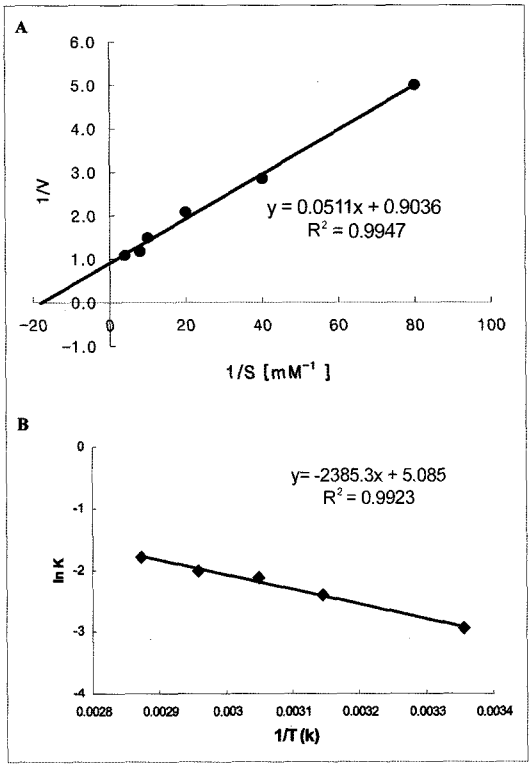

Fig. 4. Enzyme kinetic using Lineweaver-Burk plot (A) and thermal inactivation using Arrenius plot (B). A) The enzyme kinetics of actinidine (20 μg) was measured using various concentrations of pNPE substrate (0.0025-0.25 mM) in 20 mM sodium phosphate at pH 7. B) Thermal inactivation of actinidine as determined by Arrenius plot at 25, 45, 55, 65, and 75°C for 30 min.

differences in protein purity and activity (21). However, the kcat/Km value, representing the catalytic efficiency of the enzyme for the specific substrate, was 1.2 μM/sec which is similar to the value of 1.1-1.3 μM/sec obtained from other studies (12, 16, 20). Since the kcat/Km value is the most reliable measure of enzyme specificity, our enzyme shows similar specificity toward pNPE in comparison to other studies (12, 16, 20).

Thermal inactivation The thermal stability of actinidine was determined using an Arrenius plot (Fig. 4B). Actinidine is stable at 25°C since it required 90 min to become completely lost its activity. A time of 50 min is needed to completely inactivate the enzyme at 45°C. The enzyme becomes inactive at 55, 65, or 75°C with incubation times of 37, 32, or 25 min, respectively. Actinidine incubated at 45°C lost more than 70% of its activity after 6 min incubation, whereas at 55°C more than 92% of its activity was last after 18-24 min of incubation. From the slopes of the lines at each temperature, inactivation rate constants were calculated and plotted in an Arrenius plot ($y = -2385.3 x + 5.085$, R^2=0.99) (22). The activation energy for actinidine was 19.8 kJ/mol. At temperatures greater than 45°C, inactivation could be the result of protein unfolding. The high activation energy implies that the rate of this process is strongly temperature dependent, and thus at lower temperatures this rate becomes insignificant.

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References

1. Arcus AC. Proteolytic enzyme of *Actinidia Chinensis*. Biochim. Biophys. Acta 33: 242-244 (1959)
2. Boland MJ, Hardman MJ. Kinetic studies on the thiol protease from *Actinidia Chinensis*. FEBS Lett. 27: 282-284 (1972)
3. Sugiyama S, Ohtsuki K, Kawabata M. Purification and characterization of six kiwifruit proteases isolated with two ion-exchange resins, Toyopearl-Super Q and Bakerbond WP-PEI. Biosci. Biotechnol. Biochem. 12: 1994-2000 (1996)
4. Matulis D, Wu C, Pham TV, Guy C, Lovrien R. Protection of enzymes by aromatic sulfonates from inactivation by acid and elevated temperatures. J. Mol. Catal. B-Enzym. 7: 21-36 (1999)
5. Reid JD, Hussain S, Sreedharan SK, Bailey TS, Pinitglang S, Thomas EW,Verma CS, Brocklehurst K. Variation in aspects of cysteine proteinase catalytic mechanism deduced by spectroscopic observation of dithioester intermediates, kinetic analysis and molecular dynamics simulations. Biochem. J. 357: 343-352 (2001)
6. Kamphuis IG, Drenth J, Baker EN. Thiol proteases: comparative studies based on the high-resolution structures of papain and actinidine, and on amino acid sequence information for cathepsin B and H and stem bromelain. J. Mol. Biol. 182: 317-329 (1985)
7. Mcdowell MA. Anionic proteinase from *Actinidia Chinensis*. Eur. J. Biochem. 14: 214-221 (1973)
8. Pastorello EA, Conti A, Pravettoni V, Farioli L, Rivolta F, Ansaloni R, Ispano M, Incorvaia C, Giuffrida MG, Ortolani C. Identification of actinidine as the major allergen of kiwi fruit. J. Allergy Clin. Immunol. 101: 531-537 (1998)
9. Carne A, Moore CH. The amino acid sequence of the tryptic peptides from actinidine, a proteolytic enzyme from the fruit of *Actinidia Chinensis*. Biochem. J. 173: 83-83 (1978)
10. Tello-Solis SR, Valle-Guadarrama MV, Hernandez-Arana A. Purification and circular dichroism studies of multiple forms of actinidine from *Actinidia Chinensis* (kiwifruit). Plant Sci. 106: 227-232 (1995)
11. Salih E, Malthouse JPG, Kopwlessur S, Jarvis M, O'Driscoll M, Broklehurst K. Differences in the chemical and catalytic characteristics of two crystallographically identical enzyme catalytic sites. Biochem. J. 247: 181-193 (1987)
12. Lewis D, Luh BS. Development and distribution of actinidin in kiwifruit (*Actinidia Chinensis*) and its partial characterization. J. Food Biochem. 12: 109-116 (1988)
13. Patel M, Kayani IS, Mellor GW, Sreedharan S, Templeton W, Thomas EW, Thomas M, Brocklehurst K. Variation in the P2-S2 stereochemical selectivity towards the enantiomeric N-acetylphenylalanylglysine 4-nitronilides among the cysteine proteinases papin, ficin and actinidine. Biochem. J. 281: 553-559 (1992)
14. Topham CM, Salih E, Frazao C, Kowlessur D, Overington JP, Thomas M, Brocklehurst SM, Patel M, Thomas EW, Brocklehurst K. Structure-function relationships in the cysteine proteinases actinidine, papin and papaya proteinase Ω. Biochem. J. 280: 79-92 (1991)
15. Hussain S, Pinitglang S, Bailey TSF, Reid JD, Noble MA, Resminin M, Thomas EW, Greaves RB, Vermal CS, Brocklehurst K. Variation in the pH-dependent pre-steady-state and steady-state kinetic characteristics of cysteine-proteinase mechanism: evidence for electrostatic modulation of catalytic-site function by the neighbouring carboxylation anion. Biochem. J. 372: 735-746 (2003)
16. Boland MJ, Hardman MJ. The actinidine-catalyzed hydrolysis of N-α-benzyloxycarbonyl-L-lysine-*p*-nitrophenyl ester: pH dependence and mechanism Eur. J. Biochem. 36: 575-582 (1973)
17. Anthon GE, Barrett DM. Kinetic parameters for the thermal inactivation of quality-related enzymes in carrots and potatoes. J.

Agric. Food Chem. 50: 4119-4125 (2002)
18. Salih E, Broklehurst K. Investigation of the catalytic site of actinidine by using benzofuroxan as a reactive probe with selectivity for the thiolate-imidazolium ion-pair systems of cysteine proteinases. Biochem. J. 213: 713-718 (1983)
19. Sumner IG, Vaughan A, Eisenthal R, Pickersgill RW, Owen AJ, Goodenough PW. Kinetic analysis of papaya proteinase Ω. Biochim. Biophys. Acta 1164: 243-251 (1993)
20. Brocklehurst K, Baines BS, Malthouse JPG. Differences in the interactions of the catalytic groups of the active centres of actinidine and papain. Biochem. J. 197: 739-746 (1981)
21. Kim KY, Ustadi, Kim SM. Characteristics of the protease inhibitor purified from chum salmon (*Oncorhynchus keta*) eggs. Food Sci. Biotechnol. 15: 28-32 (2006)
22. Lee OH, Kang SN, Lee BY. Rheological properties of dandelion root concentrations by extraction solvents. Food Sci. Biotechnol. 15: 33-38 (2006)