Pectinesterase Inhibitor in Jelly Fig (*Ficus awkeotsang* Makino) Achenes

C.-M. JIANG, Y.-J. LAI, W.-H. CHANG, M.-C. WU, AND H.-M. CHANG

ABSTRACT: Intact and crushed jelly fig (*Ficus awkeotsang* Makino) achenes were extracted for various periods of time, and the changes in pectinesterase (PE) activities were determined. The activity of crude PE solution from intact achenes increased gradually, reaching a maximum (12 U/mL) at approximately 12 h, while the PE from crushed achenes was maintained at about 0.2 to 0.3 U/mL throughout the extraction. However, a sharp decline in PE activity (0.3 U/mL) of crude PE solution from intact achenes was observed when extract from crushed achenes was added. Heating in 100 °C water did not affect the inhibition (95% to 97%) of crude extract from crushed achenes (PE Inhibitor extract) on pea-pod (*Pisum sativum* L.) shell PE activity.

Key Words: *Ficus awleotsang* Makino achenes, pectinesterase, pectinesterase inhibitor, thermal treatment

Introduction

PECTINESTERASES (PECTIN PECTYL-HYDROLASE, EC 3.1.1.11) (PEs) have been found in higher plants and are also produced by certain plant pathogenic fungi and bacteria. By catalyzing the deesterification of pectin, plant PEs participate in the conversion of protopectin to soluble pectin and pectate, and they are important in the fruit maturation process. PEs exist in plant roots, stems, leaves, and fruits, and they have been reported to be bound with cell walls through ionic bonds (Jansen and others 1960a, 1960b).

Jelly fig (*Ficus awleotsang* Makino) is a native woody vine in Taiwan. Jelly curd from water extract of jelly fig achenes in the presence of calcium is locally popular as a summer drink. Pectinesterase (PE) from jelly fig achenes is a monomer of polypeptide with either molecular weight of 38 kD (Lin and others 1989) and isoelectric point (pI) of 3.5 or with molecular weight of 42 kD and pI of 4.5 (Komae and others 1990). This enzyme functions optimally at pH 6.5 to 7.5 when the assay mixture contains no NaCl. The optimal pH shifts to lower pH as the NaCl level increases (Lin and others 1989). The very low pI is supposed to be 1 of the features of jelly fig achene PE that differs from other PEs from higher plants (Brady 1976; Delinc'ee 1976).

Lin and others (1990) have indicated that approximately 98% of jelly fig PE exists in the pericarp of achenes, which is involved in the jellification of water extract of jelly fig achenes. However, in a preliminary observation, we found that PE activity was almost eliminated when crushed achenes were extracted to prepare jelly curd. Therefore, unknown substances with anti-PE activity were possibly extracted from the crushed achenes.

Recently, Balestrieri and others (1990) reported that a glycoprotein in ripe kiwi fruit markedly inhibited the activity of pectin methylesterase (PME) from orange, tomato, apple, banana, and potato. The inhibition of tomato PME by kiwi PME inhibitor was approved to be noncompetitive (Marquis and Bucheli 1994). In addition, the purified glycoprotein was immobilized on the resin and used as an affinity matrix in the column to separate and determine the residual pectin methylesterase activity in thermally treated vegetable products (Giovane and others 1996; Castaldo and others 1997).

Of all the inhibitors in plants, soybean trypsin inhibitors have been studied the most. Some inhibitors for pancreatic amylase and lipase have also been isolated from legumes (Schwimmer 1981). It has also been proposed that amylase inhibitors may retard the degradation of starch in the diet to provide a basis for weight control in obese individuals (Schwimmer 1981). Inhibitors that have received the most attention are the Kunitz inhibitor and the Bowman-Birk inhibitor. The latter appears to be more stable to heat denaturation than the former (Kassel 1970; Birk 1976). The loss of enzyme activity was not observed when the Bowman-Birk inhibitor was heated in the dry state to 105 °C or in 0.02% aqueous solution to 100 °C for 10 min, however, autoclaving for 20 min at 121 °C destroyed its activity (Kassel 1970; Birk 1976).

In this study, PE activities in 4% NaCl extracts prepared from intact and crushed jelly fig achenes were compared initially to ensure the existence of PE inhibitor. Subsequently, some characteristics such as thermal stability of crude PE inhibitor extracts were determined, and finally, inhibitory effects of heated PE inhibitor extract on the PE activity from pea-pod shell were studied.

Materials and Methods

Preparation of Pea-pod PE

Fresh pea-pod (*Pisum sativum* L.) shells (500 g) obtained from a local market were homogenized with a cycle blender (Osterizer Co., Berlin, Germany) at the "blend" speed for 2 min in 2 L of cold (4 °C) deionized water. The homogenization procedure was repeated for 4 times, and the mixture was filtered through 10-fold cheesecloth to obtain the residues. Extractions of PE from the pea-pod residues obtained were conducted twice with 4 and subsequently 3 volumes of 0.3 M NaCl/0.01 M phosphate buffer (pH 8.0). Filtrates obtained by filtration through 10-fold cheesecloth from both extractions were then centrifuged (14000 × g, 4 °C, 30 min) to obtain the crude pea-pod PE solution (10.0 U/mL).

The Presence of PE Inhibitor

For the determination of the release of PE inhibitor, 5 g of intact and homogenized (cycle blender, 2 min) jelly fig

achenes were stirred in a 250-mL beaker with 75 mL of 4 % NaCl at 4 °C for 0 to 96 h and 0 to 8 h, respectively (Lin and others 1989). PE activity in solutions during extraction were determined after centrifugation (5000 × g, 20 min, 4 °C) with the method described below. Triplicate samples were each analyzed twice for PE activity.

Effect of Homogenate of Crushed Achenes on PE Activity from Pea-pod Shell

Five grams of intact achenes were gently stirred in a 250-mL beaker with 75 mL of 4% NaCl at 4 °C for 12 h to obtain the crude jelly fig PE solution after filtration through a 6-fold cheesecloth. The residual achenes were then homogenized (cycle blender, 2 min) and extracted with 75 mL of 4% NaCl for 12 h followed by centrifugation (5000 × g, 20 min, 4 °C) to obtain the supernatant. Equal volumes of crude jelly fig PE solution and the supernatant obtained were mixed well with a Vortex mixer and then incubated at ambient temperature (25 to 30 °C) for 10 min. PE activities in crude jelly fig PE solution and supernatant, and residual PE activity in the mixture were determined. Crude jelly fig PE solution diluted with an equal volume of distilled water was used as a blank.

Five grams of achenes were directly crushed and homogenized (cycle blender, 2 min) in 75 mL of cold (4 °C) 4% NaCl and then incubated at 4 °C for 12 h with gentle stirring (with a magnetic stirrer). Centrifugation (5000 × g, 20 min, 4 °C) was subsequently conducted to obtain the supernatant prior to PE activity determination. Triplicate samples were each analyzed twice for PE activity.

Preparation and Thermal Stability of PE Inhibitor Extract

Initially, 5 g of intact achenes were gently stirred in 300 mL of tap water at ambient temperature (25 to 30 °C) for 1 h, and then the achenes were dried at 37 °C. Removal of pectin and jelly fig PE was further performed in 75 mL of 4% NaCl for 2 h to avoid the turbidity caused by pectin and inaccurate determination of PE activity. Achenes dried again at 37 °C were then homogenized (cycle blender, 2 min) in 75 mL of distilled water to extract (4 °C, 12 h) the PE inhibitor. The supernatant obtained by centrifugation (14000 × g, 4 °C, 20 min) was used as PE inhibitor extract.

PE inhibitor extract was incubated in 100 °C water for 5, 10, 15, 30, 45, and 60 min and then cooled in an ice bath prior to mixing with an equal volume of pea-pod PE solution. Residual pea-pod PE activity was determined after incubation for 30 min at ambient temperature (25 to 30 °C).

Pea-pod PE solution was mixed with PE inhibitor extract at various ratios (1:1, 2:1, 3:1, 4:1, 6:1, 8:1, and 10:1), with or without heating in 100 °C water for 20 min, and the residual PE activity was measured after incubation at ambient temperature (25 to 30 °C) for 30 min. PE inhibition was calculated by the following equation: PE inhibition (%) = 100% - (residual PE activity in mixture/PE activity in the starting solution) × 100%. Triplicate samples were each analyzed twice for PE activity.

Determination of Protein

Protein concentrations in solutions containing PE and PE inhibitor were assayed by the Bradford (1976) method using Bio-Rad protein assay dye reagent. Bovine serum albumen (0.2 to 1.4 mg/mL) was used to construct the standard curve.

Determination of PE Activity

PE activity was determined according to the method described by Lee and MacMillan (1968) with minor modifications. One mL of enzyme solution was added to 15 mL of 0.1M NaCl/0.5% citrus fruit pectin (degree of esterification = 68%; Sigma Chemical Co., St. Louis, Mo., U.S.A.) solution (25 °C), with the pH brought to 6.5 immediately before assay. The activity of PE was measured by titrating (pH M83 Autocal pH meter, TTT 80 titrator, ABU80 autoburette; Radiometer Copenhagen Co., Denmark) the free protons dissociated from the free carboxyl groups formed by the PE activity. The volumes (mL) of 0.01 N NaOH consumed to maintain a pH of 6.0 of the reaction solution at 25 °C (in a water bath) were recorded within 10 min. An activity unit represents 1 μeq of the free carboxyl groups produced by the PE hydrolytic activity on the pectin substrate per min at 25 °C. An enzyme solution previously heated in 100 °C water for 5 min was used as a blank. Triplicate samples were each analyzed twice.

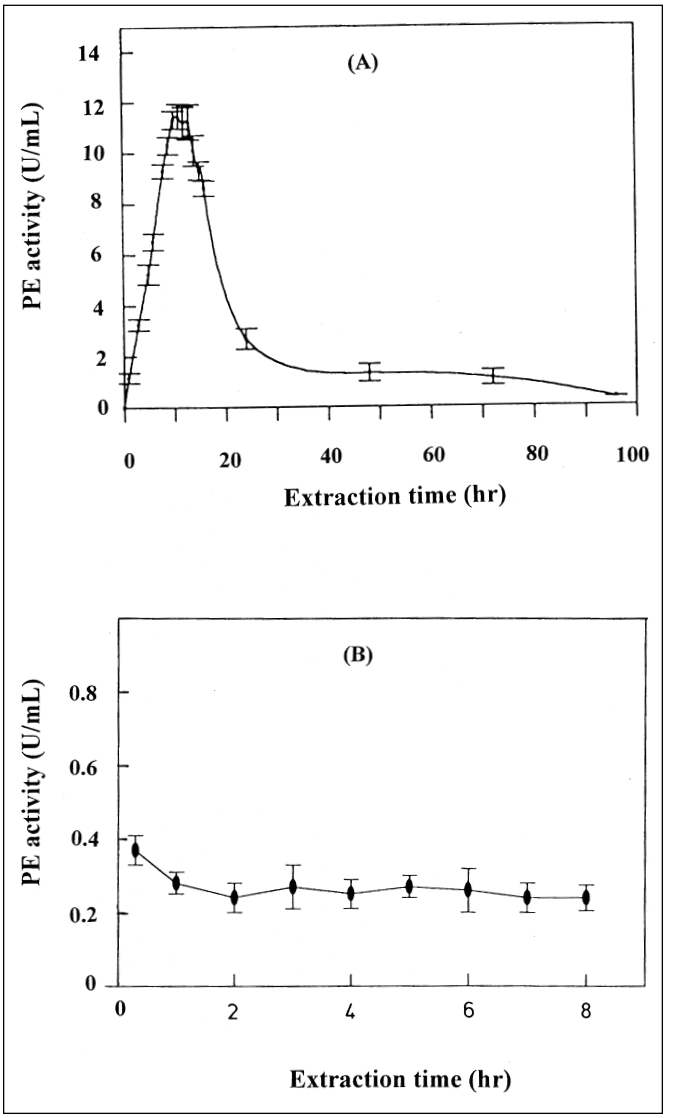

Figure 1—Changes in pectinesterase activity in 4% NaCl solutions from jelly fig intact (A) and crushed (B) achenes (5 g) during extraction with 75 mL NaCl solution for various periods of time. Each value is the average of 3 determinations.

Results and Discussion

Presence of PE Inhibitor

PE activity in the solutions prepared from intact and homogenized achenes (5 g each) with 75 mL of 4% NaCl was determined, and the results are presented in Fig. 1A and 1B, respectively. PE activity in the solution from intact achenes increased gradually to a maximal value (about 12 U/mL) after 12 h extraction and then reduced to about 3 U/mL after 24 h and to 0.2 U/mL after 90 h extraction of PE. It was observed that some of the swollen achenes were crushed during the extraction. However, much less PE activity (about 0.2 to 0.4 U/mL) in the homogenized achene solution was determined throughout the PE extraction procedure. The low PE activity in the extract from homogenized achenes could be generally due to the denaturation of enzyme as a result of homogenization, extraction, and/or the possible presence of PE inhibitor.

To determine the possible presence of PE inhibitor, intact achenes from which pectin and PE were previously removed with tap water and 4% NaCl solution were homogenized to obtain supernatant by centrifugation, with which an equal volume of crude jelly fig PE solution was mixed. It was observed that the PE activity in this mixture (Fig. 2C) was very low (about 0.3 U/mL), while that in the blank (Fig. 2D) was about 5.8 U/mL (Fig. 2). The PE activity (0.4 U/mL) in the extract prepared from homogenized achenes (Fig. 2E) was almost the same as that in crude jelly fig PE-supernatant mixture (Fig. 2C). Thus, the existence of anti-PE activity substances released from crushed achenes is clear.

Thermal Stability of PE Inhibitor Extract

PE inhibitor extract was initially heated at 100 °C in a water bath for 0 to 60 min, and then its effect on crude pea-pod PE activity was determined (Fig. 3). It was clear that no remarkable decline (95% to 97%) in PE inhibition was observed

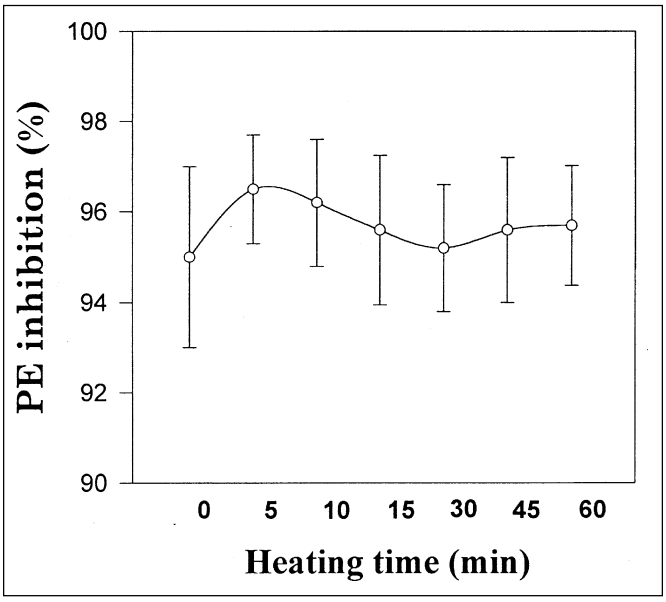

Figure 3—Changes in pectinesterase inhibition (%) of the pectinesterase inhibitor extract heated in 100 °C water for 0 to 60 min. Pectinesterase activity was determined by using crude pectinesterase (10.0 U/mL) from pea-pod shell and citrus fruit pectin (DE = 68 %) as enzyme source and substrate, respectively. Each value is the average of 3 determinations. PE inhibition (%) = 100% - (residual PE activity in mixture/PE activity in the starting solution) × 100%.

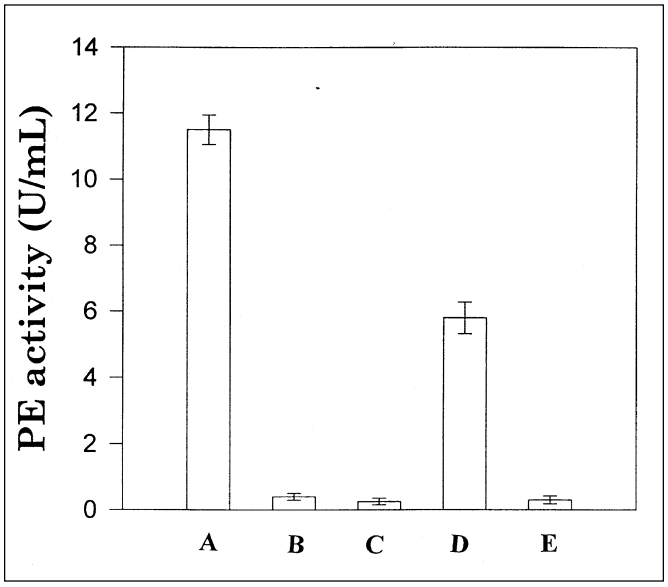

Figure 2—Pectinesterase activity in 4% NaCl solutions prepared from jelly fig intact and crushed achenes. Each value is the average of 3 determinations.
A: crude pectinesterase solution extracted from 5 g intact achenes in 75 mL 4% NaCl (extraction time = 12 h)
B: extract from crushed residues of A
C: mixture of equal volumes of A and B
D: mixture of equal volumes of A and distilled water (control)
E: homogenate of 5 g achenes in 75 mL 4% NaCl

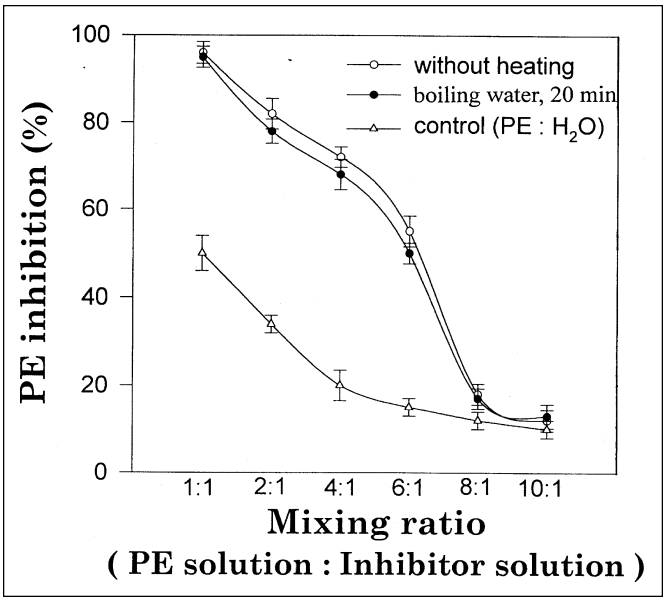

Figure 4—Comparison of the pectinesterase inhibition (%) of pectinesterase inhibitor extract with or without heating treatment in 100 °C water for 20 min on the crude pea-pod pectinesterase activity (10.0 U/mL). Crude pectinesterase from pea-pod shell was mixed with pectinesterase inhibitor extract and distilled water (control) at various ratios. Each value is the average of 3 determinations. PE inhibition (%) = 100% - (residual PE activity in mixture/PE activity in the starting solution) × 100%.

during thermal treatment for up to 60 min.

The biochemical nature of the glycoprotein inhibitor of pectin methylesterase in kiwi fruit was thoroughly studied (Balestrieri and others 1990). The amino-acid composition of it showed a very high content of acidic residues (pI 3.5), while the sugar portion is composed of galactose, arabinose, and rhamnose (Balestrieri and others 1990). The heat resistance of this glycoprotein has not been reported yet. Thus, comparison on characteristics of those 2 inhibitors appears to be impossible. However, the high thermal stability of PE inhibitor from jelly fig achenes was considered to be closely related to its biochemical composition.

PE inhibitor extract, with or without thermal treatment (100 °C, 20 min), was mixed with pea-pod shell PE solution at various ratios, and the mixtures were incubated at ambient temperature (25 to 30 °C) for 30 min prior to PE activity determination. Results shown in Fig. 4 indicate that PE inhibition decreased with the increasing ratios of pea-pod PE solution to PE inhibitor extract regardless of the thermal treatment of PE inhibitor extract. Hence, heating treatment was found to be almost without effect in reducing the PE inhibitor activity. Therefore, it was clear that the PE inhibitor derived from jelly fig achenes was also effective in decreasing the PE activity from pea-pod shell (Fig. 4), suggesting the existence of apparent cross-reactions of jelly fig PE between species. The glycoprotein inhibitor of PME in kiwi fruit was tested to be effective in inhibiting the PME from orange, tomato, apple, banana, and potato (Balestrieri and others 1990).

Conclusions

PE INHIBITOR RELEASED FROM JELLY FIG ACHENES APPARENTLY decreased the PE activity in jelly fig extracts, therefore, resulting in a softening of jelly fig curd texture during the preparation of jelly fig extract. The remarkable heat resistance of PE inhibitor extract and the apparent cross-reactions between jelly fig PE inhibitor and pea-pod shell PE reveal the possible utilization of such PE inhibitor for the fruit and vegetable industries. Since the glycoprotein inhibitor of PME in kiwi fruit was successively purified by a single-step procedure based on affinity chromatography (Giovane and others 1995), the isolation and identification of PE inhibitor from jelly fig achenes appear to be possible.

References

Balestrieri C, Castaldo D, Giovane A, Quagliuolo L, Servillo LA. 1990. Glycoprotein inhibitor of pectin methylesterase in kiwi fruit (*Actinidia chinensis*). Eur J Biochem 193(1):183-187.

Birk Y. 1976. Proteinase inhibitors from plant sources. In: Lorand L, editor. Methods in enzymology. Part B, Vol. 45. New York: Academic Press. P 659-739.

Bradford MM. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72(2):248-254.

Brady CJ. 1976. The pectinesterase of the pulp of the banana fruit. Aust J Plant Physiol 3(2):163-167.

Castaldo D, Laratta B, Loiuduce R, Giovane L, Quagliuodo L, Servillo L. 1997. Presence of residual pectin methylesterase activity in thermally stabilized industrial fruit preparations. Lebensm-Wiss U Technol 30(5):479-484.

DelincÕee H. 1976. Thin-layer isoelectric focusing of multiple forms of tomato pectinesterase. Phytochem15(7):903-907

Giovane A, Balestrieri C, Quagliuolo L, Castaldo D, Servillo L. 1995. Purification by affinity chromatography and evidence of a ripening-related precursor. Eur J Biochem 233(3):926-929.

Giovane A, Laratta B, Loiudice R, Quagliuolo L, Castaldo D, Servillo L. 1996. Determination of residual pectin methylesterase activity in food products. Biotechnol Appl Biochem 23(2):181-184.

Jansen EF, Jang R, Bonner J. 1960a. Binding of enzymes to avena coleoptile cell wall. Plant Physiol 35(5):567-571.

Jansen EF, Jang R, Bonner J. 1960b. Orange pectinesterase binding and activity. J Food Res 25(1):64-69.

Kassel B. 1970. Naturally occurring inhibitors of proteolytic enzymes. In: Perlman GE, Lorand L, editors. Methods in enzymology (proteolytic enzymes). Vol. 19. New York: Academic Press. P 839-906.

Komae K, Sone K, Kakuta M, Misaki, A. 1990. Purification and characterization of pectinesterase from *Ficus awkeotsang*. Agric Biol Chem 53(5):1237-1241.

Lee M, MacMillan JD. 1968. Mode of action of pectin enzymes. I. Purification and certain properties of tomato pectinesterase. Biochem 7(11):4025-4030.

Lin TP, Liu CC, Chen SW, Wang WY. 1989. Purification and characterization of pectinmethylesterase from *Ficus awkeotsang* Makino achenes. Plant Physiol 91(4):1445-1453.

Lin TP, Liu CC, Huang RS, Wang, WY, Fen TY. 1990. Induction of pectin methylesterase in the pericarp of achenes of the jelly-fig *Ficus awkeotsang* Makino. Plant Cell Physiol 31(4):533-539.

Marquis H, Bucheli P. 1994. Inhibition of tomato pectin methylesterase by partially purified kiwit pectin methylesterase inhibitor protein. Int J Food Sci Technol 29(2):121-128.

Schwimmer S. 1981. Source book of food enzymology. Westport, Conn: APV Publishing Co. p. 121-128.

Ms. 20000234

We are grateful to Mr. Wun-Hong Lee, a jelly fig achenes dealer in Pingtung County, for supplying samples of jelly fig achenes.

Author Jiang is with the Dept. of Food and Beverage Management, National Kaohsiung Hospitality College, Kaohsiung 800, Taiwan. Authors Lai, W.-H. Chang and H.-M. Chang are with the Graduate Institute of Food Science and Technology, National Taiwan Univ., Taipei 106, Taiwan. Author Wu is with the Dept. of Food Science, National Pingtung Univ. of Science and Technology, Pingtung 91201, Taiwan. Direct correspondence to Hung-Min Chang (E-mail: changhm@ccms.ntu.edu.tw).