Kasetsart J. (Nat. Sci.) 39 : 125 - 135 (2005)

Inhibitory Effects of Mulberry Leaf Lectins to Silkworm Proteases

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ABSTRACT

Proteases from digestive fluid of Nang-lai strains of the Thai silkworm, *Bombyx mori* were partially purified by 40% ammonium sulfate fractionation and *p*-aminobenzidine agarose affinity chromatography. Six proteases named P1-P6 were obtained and P4, P5 and P6 were purified approximately 21,18 and 17 folds, respectively. The P4, P5 and P6 proteases had an equal apparent subunit molecular weight of 24 kDa and native molecular weight of 66 kDa. They exhibited the alkaline optimum pH at pH 9.0, 11.0 and 11.5, respectively. Their proteolytic activities were inhibited by specific trypsin inhibitors including leupeptin, tyrosyl-L-lysine chloromethylketone and soybean trypsin inhibitor suggesting that they were trypsin-like proteases. Inhibitory effect of two lectins from mulberry leaf, MLL1 and MLL2, to the proteases were studied. It was found that MLL1, in comparison to MLL2 and ConA, had greater inhibitory effects towards the protease P4 and a lesser inhibitory effect towards the protease P5 and P6. The inhibition of the proteases by both mulberry leaf lectins was not recovered by the specific lectin-binding sugars, N-glycolylneuraminic acid and N-acetylgalactosamine.

Key words: mulberry leaf lectins, silkworm proteases, inhibitory effects

INTRODUCTION

Plant lectins are carbohydrate-binding proteins widely distributed in different species of plants (Goldstein and Poretz, 1986; Etler, 1986). They are found abundant in seeds, roots, sap, fruit, flowers, barks, stem and leaves. Despite their abundance, their physiological functions are not clearly defined. They are purposed to play different roles either within or outside plants including transport of carbohydrate, packaging and/or mobilization of storage proteins and carbohydrate, cell wall elongation, interaction between plants and microorganisms and defense against the attack of fungi, virus, pests and insects (Etler, 1986; Chrispeel and Raikhel, 1996; Van Damme and Pneumans, 1995; Van Damme *et al*., 1993). Many plant lectins have been shown to have direct inhibitory effects on some digestive enzymes of higher animals and insects including α-amylases (Thompson and Gabon, 1987; Fish and Thompson, 1991), esterases and proteases (Belzunces *et al*., 1994; Thompson, *et al*., 1986). Many studies on legume seed lectins showed that they resulted in antinutritive effect and defense of the plants against insects and pathogenic organisms (Van Damme and Pneumans, 1995; Fabre, 1998). In silkworm, *Bombyx mori*, the presence of intestinal proteases has been reported (Eguchi and Iwamoto, 1976; Eguchi, 1982; Sasaki and Suzuki, 1982). Because of the digestion of mulberry leaves and quality of the alkaline proteases may make body of the

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Received date : 18/10/04 Accepted date : 02/02/05

silkworms larger and provides more silk, attention has been focused primarily on the digestive fluid proteases. It has been known that silkworm in its fifth larval instar exhibited a high alkaline proteolytic activity in the midgut digestive fluid (Eguchi and Iwamoto,1982). Two new N-glycolylneuraminic acid binding lectins, named MLL1 and MLL2 have been discovered from leaves of the mulberry (Ratanapo *et al*., 1998). Although a posssible role of the mulberry leaf lectin, MLL1 on the interaction to *Pseudomonas syringae* pv *mori*, a specific pathogenic bacteria of the mulberry leaf have been suggested (Ratanapo *et al*., 2002), the other biological functions of both lectins have not been elucidated. It was described here the effect of lectins to the alkaline proteases partially purified from digestive fluid of silkworm. The results might extend the understanding of the roles that mulberry leaf lectins play in nutrition of silkworm.

MATERIALS AND METHODS

Purification of mulberry leaf lectins

Two mulberry leaf lectins, MLL1 and MLL2 were purified from mulberry, *Morus rotunbiloba* Koidz by GalNAc-agarose affinity column chromatography, Sephacryl S-200 gel filtration column and DEAE-Sephacel anion-exchange column chromatography according to Ratanapo *et al.* (1998).

Preparation of digestive fluid from silkworm

Nang-lai strains of Thai silkworm, *Bombyx mori* were used. The fifth instar larvae at the third day after ecdysis were starved for a day and then treated with chloroform. Digestive fluid secreted was collected, pooled and centrifuged at 13,000xg for 15 min at 4°C.

Purification of alkaline proteases from digestive fluid of silkworm

Protein in digestive fluid of silkworm was precipitated with 40% saturated ammonium sulfate. The precipitate was dissolved, dialysed against 0.01 M borate-NaOH, pH 10.0 containing 1% glycerol and 60 mg protein was applied onto a *p*-aminobenzidine agarose column (1.5×10 cm) pre-equilibrated with the same buffer.The column was washed with the borate buffer at flow rate 15 ml / h until absorbance at 280 nm of effluent became zero. The column was then eluted sequentially with the buffer containing 0.1, 0.2, 0.6 and 1.0 M NaCl. Three ml fractions were collected and dialysed against the buffer overnight. Fractions exhibiting protease activities were pooled, kept at 4°C and used for further studies. Native molecular weight of each protease fraction was determined by Sephacryl S-200 gel filtration column. Molecular weight markers used were bovine serum albumin (67,000), ovalbumin (43,000), chymotrypsinogen A (25,000) and ribonuclease A (13,700).

Assay of alkaline protease activity

Trypsin-like activity of a purified protease was determined at 27°C in 1 ml reaction mixture containing enzyme, 0.3 mM benzoyl-L-arginine-*p*-nitroanilide and 0.01 M borate-NaOH, pH 10.0. Catalytic activity of the enzyme was measured by following the increase in absorbancy at 405 nm of *p*-nitroaniline at the function of time for 10 min. Blank was set under an identical condition using buffer instead of enzyme. One unit of protease activity was defined as absorbancy increased at 405 nm per min.

Effect of pH on protease activity

The optimum pH of purified proteases were determined as described above by adding 50 µl of each affinity purified proteases, P4, P5 and P6 into 750 µl of 0.01 M borate-NaOH buffer of pH 9.0-10.5 or 0.01 M phosphate-NaOH buffer of pH 11.0-12.0 for 10 min before adding the substrate.

Effect of temperature on protease activity

Temperature stability of purified protease was determined by incubating the enzyme at 20°C to 70°C for 30 min before assaying the activity.

Effect of trypsin inhibitors and MLLs on protease activity

Specific trypsin protease inhibitors including leupeptin, TLCK (tyrosyl-L-lysine chloromethylketone) and soybean trypsin inhibitor were incubated with purified protease at 4°C for 10 min. Decreasing in the catalytic activity was monitored. Effects of two mulberry leaf lectin, MLL1, MLL2 and another plant lectin, Con A (Concanavalin A), on purified protease were determined by preincubating each lectin of various concentration with fixed amount of purified protease at 4°C for 30 min. Control was performed under identical conditions using heat denatured lectin.

Sugar inhibition of MLLs-proteases interaction

To determine the inhibition of interaction between MLLs and purified proteases by lectin specific-binding sugars, NeuGc and GalNAc in 0.1 M Tris-HCl buffer saline (TBS), each sugar at various concentrations (25, 50 and 100 mM) was mixed with an equal volume of lectin-protease mixture (protein ratio of protease to lectin = 1 : 4) at 4°C for 30 min. The protease assay mixture was then added to a final volume of 1 ml. Increasing in the protease activity was followed at the function of time.

Resistance of lectins to proteolysis

The resistance of lectins, MLL1, MLL2 and Con A towards trypsin and the proteases from the digestive fluid were determined by incubating each lectin with each protease at the fixed protein / protease concentration ratio (4 : 1) for a period of time (5, 10, 15, 20, 30, 40 and 60 min) and then stopped the reaction with an equal volume of 10% trichloroacetic acid. After standing at room temperature for 30 min, precipitate was removed. Increased amounts of released amino acids and peptides in the supernatant was then measured by following the increasing of absorbancy at 570 nm by which the color developed upon ninhydrin reagent use (Laemmli, 1970).

SDS-PAGE polyacrylamide electrophoresis

Slab gel electrophoresis was performed followed the method of Laemmli (1970), using 12% polyacrylamide in the presence of 0.1% sodium dodecylsulfate. Protein bands were then detected with Coomassie Blue R-250 stain. Molecular weight markers used were bovine serum albumin (66,000), pepsin (34,700), trypsinogen (24,000), lactoglobulin (18,400) and lyzozyme (14,300).

Protein determination

Protein concentration of the protease was determined by the method of Lowry *et al*. (1951).

RESULTS

Purification and molecular characterization of alkaline proteases

Protein in digestive fluid of silkworm was fractionated by ammonium sulfate precipitation (results not shown). Most of the alkaline protease activity was recovered in 0-40% ammonium sulfate fraction. Subsequent purification by affinity chromatography of the proteases on *p*-aminobenzidine agarose column resulted in separation of the proteases into six protein peaks, named P1- P6 (Figure 1). The first peak, P1 was not trapped in the column and was washed out as an unbound fraction (no unbound protein was found when rechromatographed P1 on the *p*-aminobenzidine agarose column, data not shown). The other five retained proteases were then eluted from the column by stepwised NaCl concentration. It was found that the pool protease active fractions corresponding to P4, P5 and P6 gave high specific

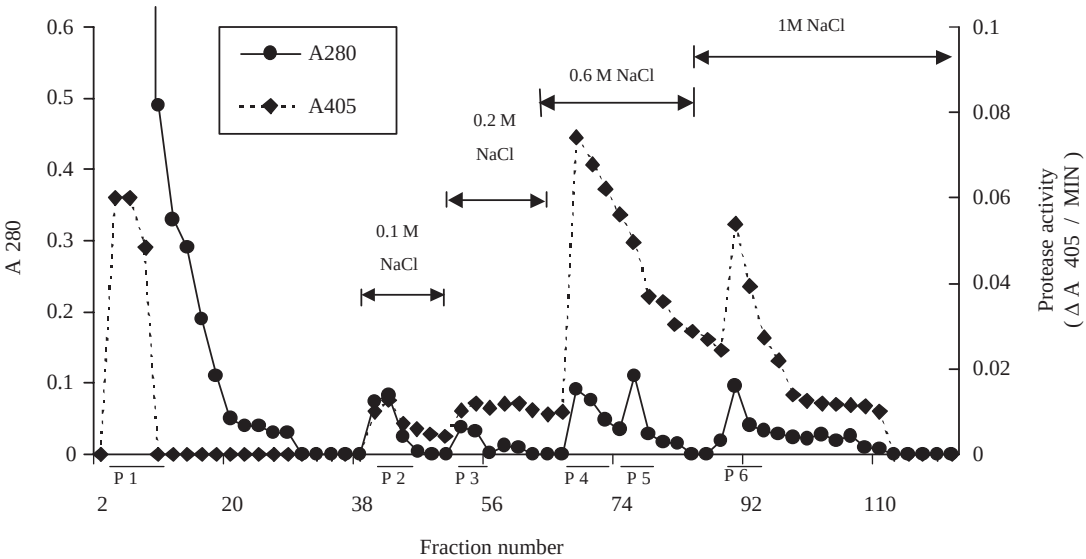

Figure 1 Affinity chromatography of alkaline proteases on *p*-aminobenzamidine – agarose column. Ammonium sulfate fraction (0– 40%) with 60 mg protein from the silkworm digestive fluid applied onto the column (1.5×10 cm), washed with 0.01 M borate-NaOH, pH 10.0 and eluted with stepwise elution using the buffer containing 0.1, 0.2, 0.6 and 1 M NaCl, respectively.

activities with approximately 21, 18 and 17 purification folds, respectively (Table 1).

Analysis of the protein pattern of each protease fraction by SDS-PAGE showed that P4, P5 and P6 were pure proteins giving an equal apparent subunit molecular weight of 24 kDa (Figure 2). Gel filtration on a Sephacryl S-200 column, each native protease was eluted with an apparent molecular weight of 66 kDa (result not shown). This suggested that P4, P5 and P6 could be a trimer and composed of identical subunits.

Thrice proteases, P4, P5 and P6 were heat labile enzymes (Figure 3). The protease activities were almost lost at 60°C. However, it seemed that the protease, P5, was more stable than the proteases, P4 and P6, because it remained 100% stable at 50° C while the others were 30-40% activity losses. Optimum pH of the three purified proteases are shown in Figure 4. The protease P4, P5 and P6 exhibited the alkaline optimum pH at pH 9.0, 11.0 and 11.5, respectively.

Effect of trypsin inhibitors on protease activities

Effects of specific trypsin inhibitor, leupeptin, TLCK and soybean trypsin inhibitor on the activities of purified protease, P4, P5 and P6 are shown in Figure 5. Their proteolytic activities were effectively inhibited by those inhibitors which soybean trypsin inhibitor at 10 µg / ml was the most potent one. The inhibition by the specific inhibitors of trypsin indicated that the purified proteases were trypsin-like proteases.

Effects of MLL on protease activities

Effects of two purified mulberry leaf lectin, MLL1 and MLL2 on the proteolytic activities of protease, P4, P5 and P6, were monitored by following the decrease in the rate of proteolytic activities after incubating the enzymes with lectins for 30 min. It was found that more increase in the lectin / enzyme protein ratios produced more inhibitory effects to protease activities as shown in Figure 6. At high concentration of lectins (lectin /

Table 1 Purification of alkaline proteases from digestive fluid of silkworm, *Bombyx mori*.

Fraction	Volume (ml)	Total protein (mg)	Total activity (ΔA405 /min)	Specific activity (A405/min/mg)	Purification fold
Crude	53.8	150.5	118.25	0.79	1
0 - 40 % (NH4)2SO4	12.0	60.0	29.28	0.49	0.62
p-Aminobenzamidine –agarose					
Unbound : P1	18.6	40.92	30.88	0.75	0.95
Bound : P2 (fraction no. 63-67)	17.0	1.28	4.05	3.16	4.00
P3 (fraction no. 79-83)	7.8	0.31	1.76	5.69	7.20
P4 (fraction no. 92-109)	10.8	0.86	14.19	16.50	20.89
P5 (fraction no. 117-121)	6.9	0.41	5.98	14.57	18.44
P6 (fraction no. 134-145)	6.5	0.38	5.25	13.82	17.49

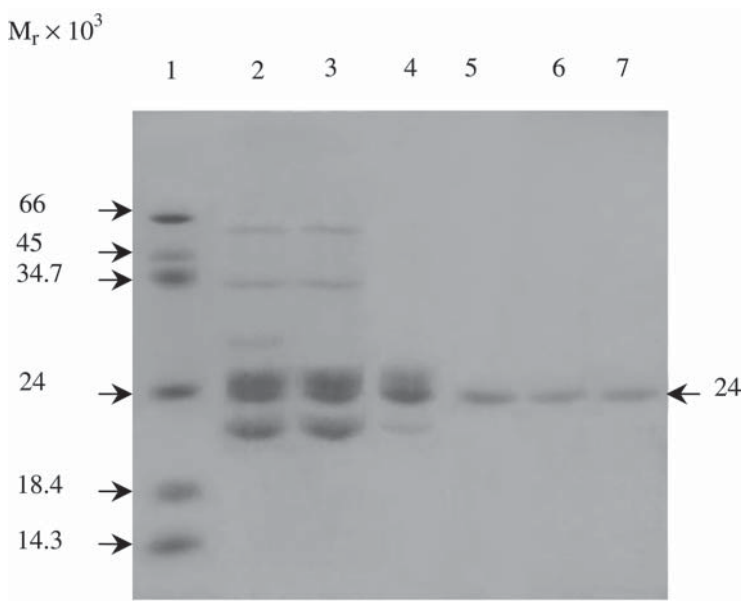

Figure 2 SDS-PAGE of the purified proteases from the silkworm using 12% polyacrylamide gel containing 1% SDS.
Lane 1, Marker proteins; Lane 2, P1 (67 µg); Lane 3, P2 (67 µg) ; Lane 4, P3 (45 µg); Lane 5, P4 (20 µg); Lane 6 P5 (20 µg); Lane 7, P6 (20 µg).

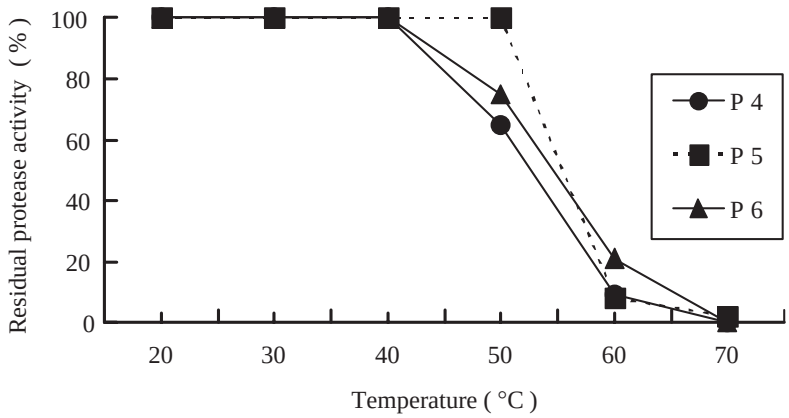

Figure 3 Temperature stability of the purified proteases from the silkworm. Each affinity purified proteases, P4, P5 and P6 incubated at 20°-70°C for 30 min before assaying the proteases activities.

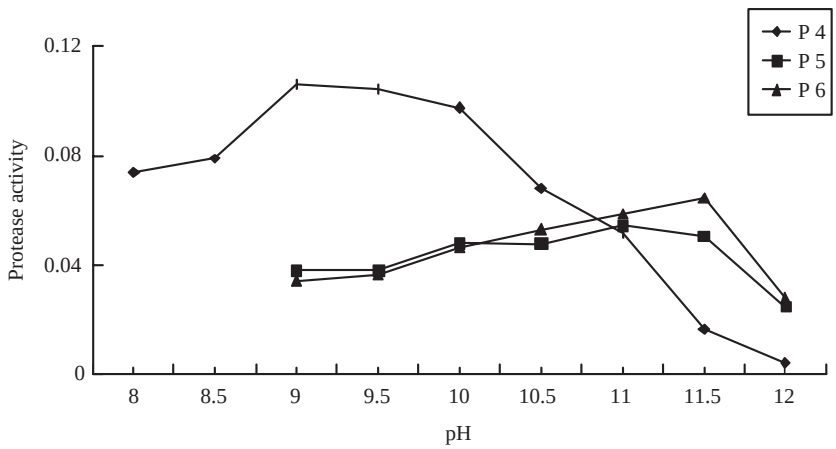

Figure 4 Optimum pH of the purified proteases from the silkworm. 50 µl of each affinity purified proteases, P4, P5 and P6 added into 750 µl of buffer of pH 8.0 –12.0 for10 min before assaying the protease activities.

enzyme protein ratio of 4:1), MLL1 had a greater reactivity towards protease P4 (32% reduction) than Con A (30% reduction) and MLL2 (28% reduction) (Figure 6a). At the same concentration, MLL1 and MLL2 showed an equal reactivity towards protease P5 (43% reduction) but greater than Con A (30% reduction) (Figure 6b). MLL2, however, had greater reactivity towards protease P6 (49% reduction) than MLL1 and Con A (43% reduction) (Figure 6c). When those lectins were heated at 100°C for 30 min before incubating with the enzymes, no reduction of proteolytic activities was observed.

The inhibitory effects of MLL to proteases, P4, P5 and P6, were not recovered by the lectin-binding sugars, NeuGc at concentration up to 50 mM or GalNAc at the concentration up to 100 mM (result not shown).

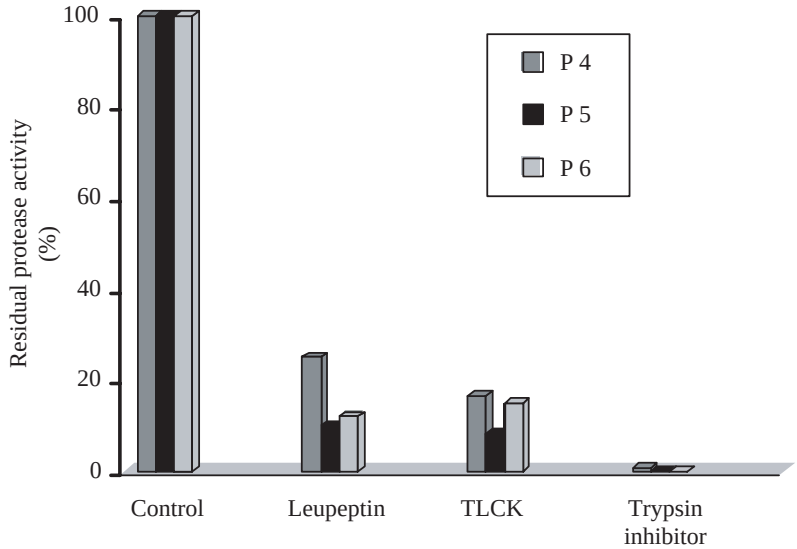

Figure 5 Effects of trypsin inhibitor on the purified proteases from the silkworm. Each affinity purified protease (P4, P5, P6) from the silkworm incubated with trypsin inhibitors, leupeptin (10 µg / ml), TLCK (5mM) and soybean trypsin inhibitor (10 µg / ml) for 10 min before assaying the protease activities.

Resistance of lectins to proteolysis

Time course of the proteolysis of MLLs by silkworm proteases was monitored in comparison to the proteolysis of a standard protein, casein. MLL1 and MLL2 were more resistant to proteases than casein (Figure 7).

DISCUSSION

Mulberry leaf lectins, MLL1 and MLL2 are unique plant lectins that exhibit sugar binding activity towards N-glycolylneuraminic acid which mostly is absent in the plant but is a carbohydrate component of animal glycoproteins (Schauer, 1982). The anti-insect activity of some plant lectins have been reported on the basis of their inhibitory effects on larval growth and development (Murdock *et al.*, 1990; Czapla and Lang, 1990; Huesing, 1991). Although mechanisms of those plant lectin action were not exactly known, the binding of lectins to glycoconjugates exposed on the epithelial cells along the digestive tract of insects or binding of lectins to glycosylated digestive enzymes seem to be possible. Red kidney bean lectin and Con A have been shown to reduce the rate of starch digestibility of porcine pancreatic amylase and human salivary amylase by non-competitive inhibition (Thompson and Gabon, 1987). Both lectins produced antinutrient effects by reducing protein hydrolysis activities of pepsin and pancreatin (Thompson *et al*., 1986). Recently, wheat germ agglutinin has been shown *in vitro* to have no effect on the activities of trypsin and non-specific protease from midgut of bee, *Apis mellifera*, but it elicited a large decrease in trypsin activity *in vivo* (Fabre, 1998). Alkaline proteases in digestive juice and midgut of the silkworm, *Bombyx mori* have been demonstrated (Eguchi *et al.*, 1980; Sasaki and Suzuki, 1982; Eguchi and Iwamoto, 1982) but there is no report of direct interaction of those proteases with plant lectins. It was found that proteases in the digestive fluid of Thai silkworm, Nang-lai, were trypsin-like isozymes and three of them (P4, P5 and P6) could

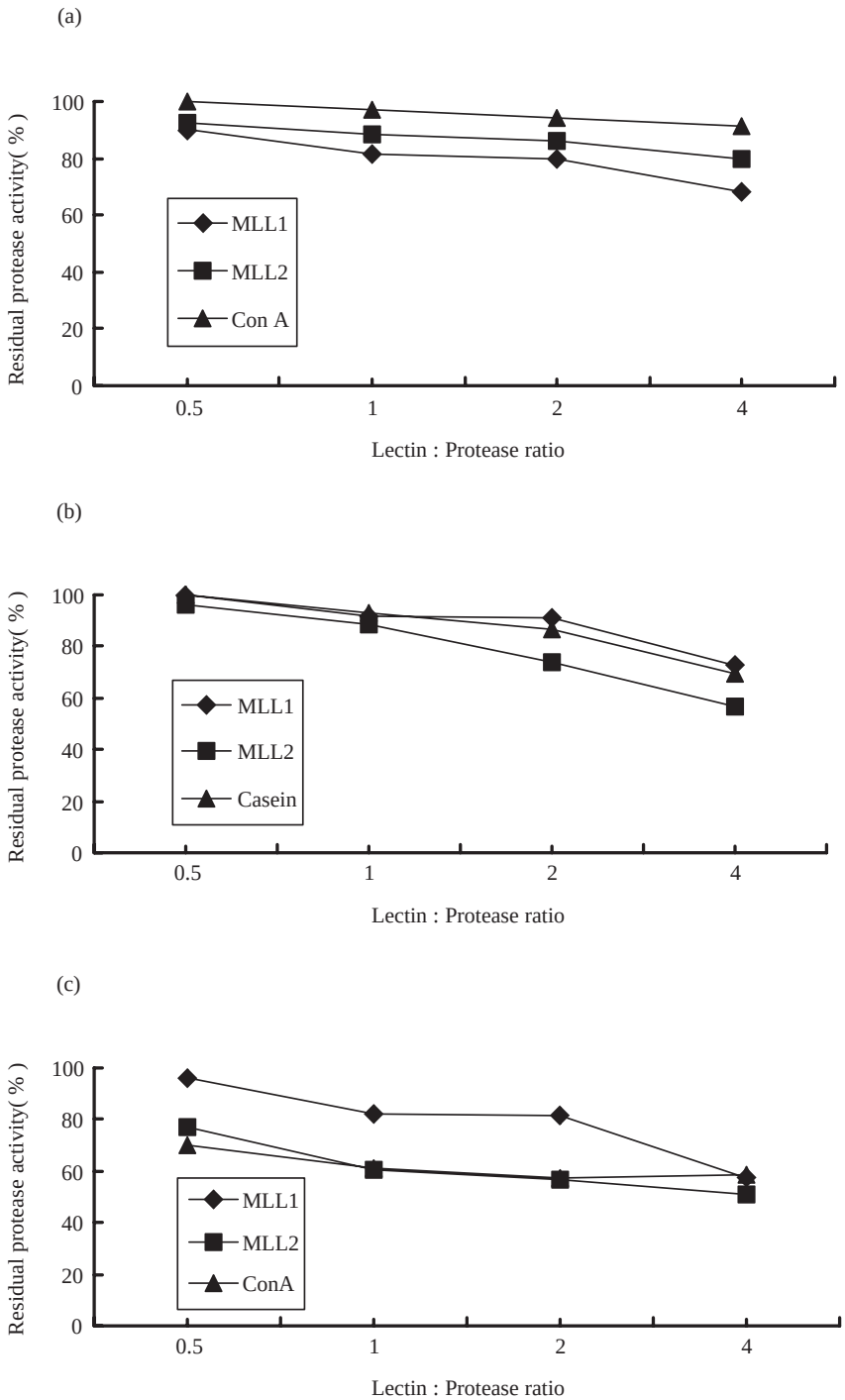

Figure 6 Effects of mulberry leaf lectin on silkworm proteases. MLL1, MLL2 and ConA each of various concentration preincubated with fixed amount of the purified protease (a) P4, (b) P5, and (c) P6 at 4°C for 30 min before assaying the protease activities.

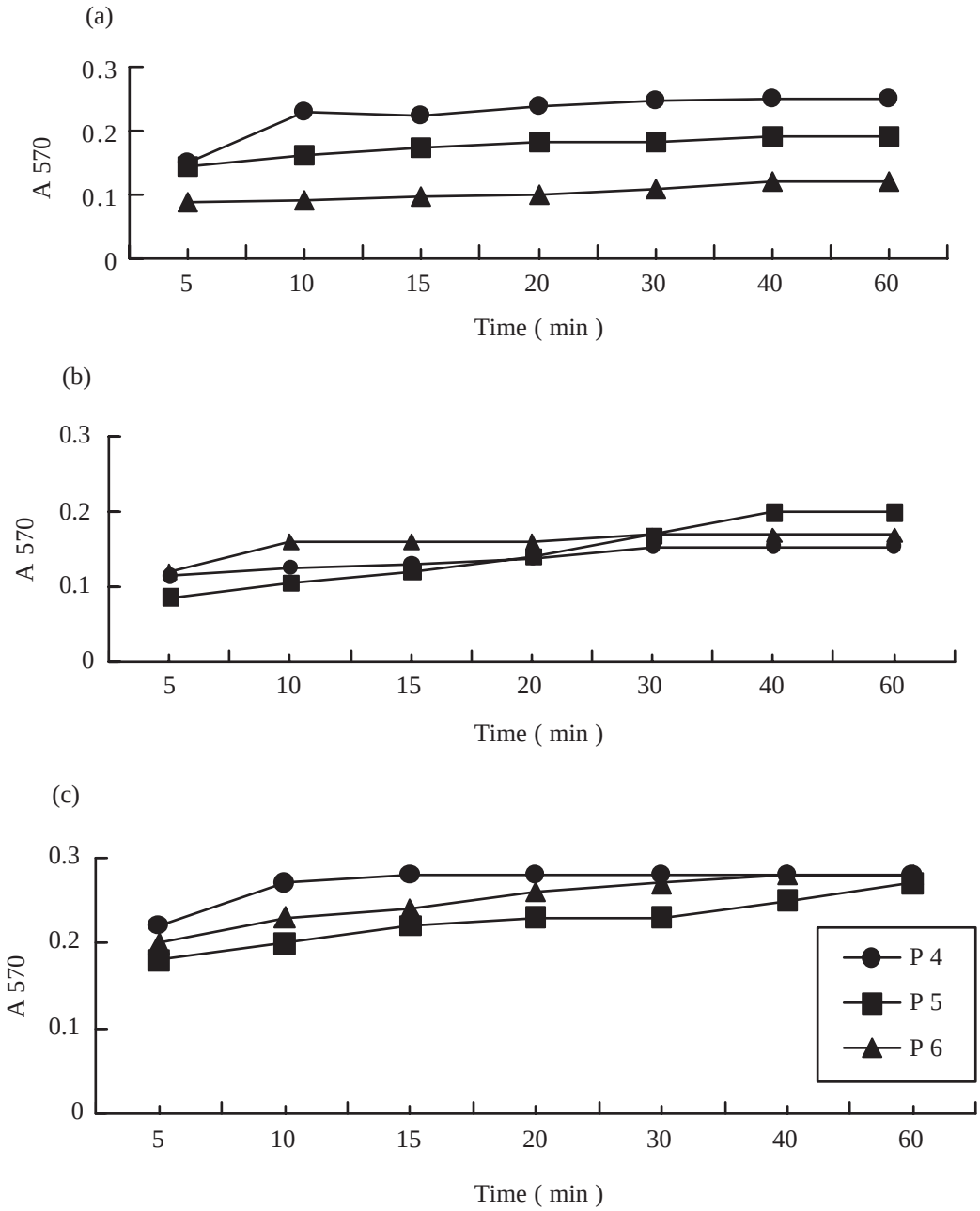

Figure 7 Proteolysis resistance of mulberry leaf lectins to silkworm proteases
MLL1 (a), MLL2 (b), and casein (c) each incubated with the purified proteases from silkworm, P4, P5 and P6 at fixed lectin / protease ratio (4:1) for a period of time and then the reaction stopped with an equal volume of 10 % TCA, the amount of amino acids and peptides released in the supernatant measured by following the absorbancy at 570 nm due to the color developed by ninhydrin reagent.

be purified by only two step separations including ammonium-sulfate fractionation and affinity chromatography using a trypsin inhibitor, *p*-aminobenzidine as ligand. The subunit molecular weights of 24 kDa of the purified proteases were closely resembled to those two alkaline proteases (PII = 22 kDa and PIII = 23 kDa) purified from the digestive juice of a hybrid silkworm, *Bombyx mori* (Sasaki and Suzuki, 1982) by using Amberlite IRC-50 ion-exchange chromatography and soybean Bowman-Birk inhibitor-Sepharose 4B affinity chromatography. The optimum pH of purified proteases, P5 and P6 were, 11.0 and 11.5, respectively, which were closely resembled to the former report of pH 10.0 –11.0 (Sasaki and Suzuki, 1982) except P4 which exhibited lower optimum pH (pH 9.0).

Pre-incubation of mulberry leaf lectins, MLL1 and MLL2 and Con A with the purified alkaline proteases from digestive fluid of the silkworm, P4, P5 and P6 resulted in a significant decrease of proteolytic activities as shown in Figure 6(a), 6(b) and 6(c), respectively. At alkaline pH of the enzyme assay (pH 10.0), hemagglutinating activities of such lectins were almost lost (data not shown). Moreover, heating the lectins abolished their abilities to inhibit the protease activities. Inhibitory activities of lectin MLL to each protease were not recovered by MLLs-binding sugars, NeuGc and GalNAc. The results suggested that inhibition of the proteases by plant lectins was not due to the binding of lectin sugar-binding sites to glycosylated part of the enzyme molecules. The inhibition may probably due to the complex formation between native lectins and proteases from digestive fluid. Kinetic study will be further evaluated to define the type of enzyme inhibition.

CONCLUSION

The present experiments demonstrate that two mulberry leaf lectins, MLL1 and MLL2 have inhibitory effects to trypsin-like alkaline proteases purified from digestive fluid of the fifth larval instar of the silkworm, *Bombyx mori*. Anti-proteolytic effect of lectins towards the digestive proteases might occur prior to the silkworm digestion of food protein. The reduction of digestive protease activities by leaf lectins might cause the body size of silkworm to become smaller and thus provided less silk. In addition to lectins, mulberry leaves might contain a wide variety of constituents such as phenolic compounds, phytate, and enzyme inhibitors which might or might not interact with lectins or digestive proteases. Thus, the individual and the related antinutrient effects of such constituents towards proteases have to be considered in silkworm cultivation.

ACKNOWLEDGEMENTS

This work was supported by Kasetsart University Research and Development Institute (KURDI) to S. Ratanapo. Special thanks go to Center of Sericulture Udornthani for providing mulberry leaves and silkworm larvae. Grateful appreciation is expressed to Prof. M. Chulavatnatol for his valuable support and advice. Sincere thanks go to Miss A.Thattiyapong for preparation of the manuscript.

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