Intellectual Contribution

Synergistic defensive function of raphides and protease

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We clarified that raphides, calcium oxalate needle crystals, exert strong insecticidic or growth-inhibiting activities against insects and function as a defense of plants against herbivorous insects by synergistically intensifying the defense activity of cysteine protease that coexist with raphides in plant tissues.

Keywords:raphide, calcium oxalate, needle crystal, cysteine protease, plant defense, plant-insect interaction

Background

Raphides, tiny needle-shaped calcium oxalate crystals, are present in large amount in tissues of many plant species including kiwifruit, pineapple, taro, yam, and grape. Although raphides may play defensive roles against herbivores, there are no direct experimental evidence showing their modes of function based on purified raphides. Since raphides frequently coincide in the same plant tissue with other defensive substances such as protease in the case of kiwifruit and pineapple, we hypothesized that raphides make holes in plant tissues and/or cell membranes and facilitate other defense substances to reach their targets, thereby intensifying the defense activity of defense substances, which can be called the needle effect. Therefore, we performed clear bioassays feeding the larvae of the Eri silkmoth (*Samia ricini*) with leaves from the host castor oil plant (*Ricinus communis*) painted with the raphides purified from kiwifruits (*Actinidia deliciosa*) in the presence or absence of cysteine protease (or other defense proteins) that often coincide with raphides in plant tissues.

Results and Discussion

1. We successfully purified raphides by homogenizing kiwifruit tissues in heavy liquid (dense CsCl solution with a specific gravity of 1.8), centrifugation of the homogenate, and collecting the precipitate with specific gravity of more than 2. The collected raphides were very sharp with a length of 0.1mm (Fig. 1).
2. A much stronger defense activity was observed in the presence of both raphides and cysteine protease than either raphides or cysteine protease only (Fig. 2, Table 1). When neonate larvae of the Eri silkmoth were fed with leaves of castor oil plant painted with either 41.7μg/cm^2 of raphides (Fig. 2B) or 0.22mg/cm^2 of cysteine protease only (Fig. 2C), the mortality rate was very low (0%) and the larvae grew nearly as well as when they were fed with unpainted castor oil plant leaves (Fig. 2A). Even when the larvae were fed with leaves painted twice the concentration of either raphides or cysteine protease, defensive activities remained weak. In contrast, when larvae were fed with leaves painted with both raphides and cysteine protease together, extremely strong defense activity was observed with larval mortality of 69% without any trace of growth, the larvae died within two hours and the body turned black and soft (Fig. 2D). These results indicated that raphides and cysteine protease exert synergistic defensive function against insect herbivores. Our results also clarified that the toxicity of cysteine protease was intensified to 16-32 times in the presence of a small amount of raphides (Table 1).
3. The needle-like shape is an essential factor in the synergistic defense effect of raphides; amorphous calcium oxalate crystal and cysteine protease did not show any synergistic effect.
4. Raphides also showed synergistic defense activities with chitinase as well as protease suggesting that raphides may function as a general intensifier of various defense substances.

Future prospects

1. Synergism between raphides and other defensive substances will facilitate a deeper understanding of the defense mechanisms of many cultivated plants that contain raphides such as kiwifruit, pineapple, taro, yam, and grape.
2. Raphides can possibly be used as an intensifier of agrochemicals and defense proteins, and the synergistic function may help in developing insect resistant crop varieties.

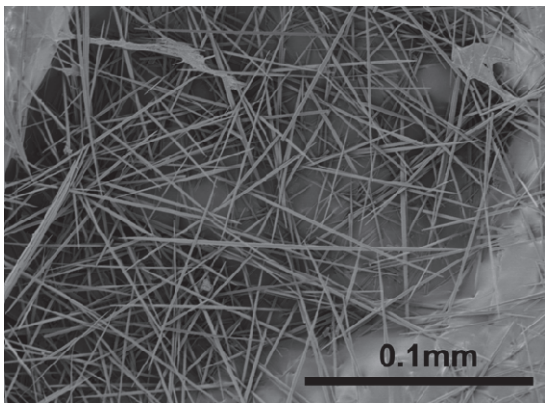

Fig. 1. Raphides, needle-shape calcium oxalate crystals that were purified from kiwifruit have very sharp shape measuring ca. 0.1 mm in length.

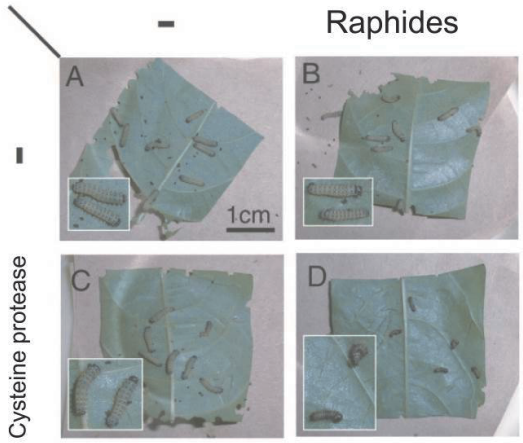

Fig. 2. Synergistic defensive function of raphides and cysteine protease against insects. Leaves painted with raphides alone, cysteine protease alone, or those painted with both are fed to the larvae of Eri silkmoth and defensive activities were compared among treatments. (A) Control (unpainted leaf), (B) Leaf painted with raphides. (C) Leaf painted with cysteine protease, (D) Leaf painted with both raphides and cysteine protease. Strong defensive activities (growth inhibition and insecticidic activity) were observed only when the leaf was painted with both raphides and cysteine protease (D).

Table 1. Numerical relationship between raphides and cysteine protease in terms of synergism defensive effects based on mortalities of Eri silkworm in day 1 (%) are indicated in the table (n=16-17, — ; not tested).

Cysteine protease (mg/cm^2) \ Raphides ($\mu g/cm^2$)	0	5.2	10.4	41.7	83.3
0	0.0	0.0	0.0	0.0	0.0
0.014	0.0	0.0	0.0	0.0	—
0.028	0.0	0.0	0.0	0.0	—
0.056	0.0	31.3	23.5	23.1	—
0.11	0.0	31.3	87.5	37.5	—
0.22	0.0	81.3	87.5	68.8	—
0.44	23.1	—	—	—	—
0.89	0.0	—	—	—	—

Reference

1. Konno K, Inoue T.A, Nakamura M (2014) Synergistic defensive function of raphides and protease through the needle effect ***PLoS ONE*** 9 (3):e91341