BIOLOGICAL ROLES AND APPLICATION OF CHITINASE

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Abstract

Chitinases are produced by many organisms to play an individual physiological role. In plants, both family 18 and 19 chitinases are produced for self-defense against pathogens and pests. In insects, only family 18 chitinases are produced for degradation of the old cuticle chitin during the ecdysis and metabolism of peritrophic membrane chitin. In order to investigate the relationship between classification based on amino acid sequence and biological role, we purified some chitinase isozymes from yam as a plant and from the silkworm as an insect, and then investigated the characteristic properties including kinetic behavior.

First we describe specially an advantage of a class IV chitinase (family 19) from yam against plant pathogens on the basis of its characteristics, where class IV is one of the classes adapted by plans. Furthermore, we describe the mechanism for production of insect chitinase at transcriptional and translational levels, such as alternative splicing and post-translational processing, respectively.

As application of chitinases to agriculture, we demonstrate some fruitful results using yam class IV chitinase and some insect chitinases. One is development of a transgenic plant resistant to plant pathogens. The other is a development of bio-control agents instead of chemical fungicides and insecticides. We also discuss on the role of chitin-binding domain additionally included in chitinase.

Introduction

In order to clarify the role of plant chitinases (EC3.2.1.14), we first investigated the induction of chitinase by inoculating yam callus with pathogens, cell-wall components of pathogen such as chitin and hormone such as ethylene [1]. The induced chitinase isozymes are dependent on the used elicitor (inducer). Among them, yam class IV chitinase, which was later identified by the analysis of partial amino acid sequence and cloning its genomic DNA, was induced by all elicitors used. Next we investigated the biological role of plant chitinase by comparison of lytic activity as a biological role with enzymatic properties using chitinase isozymes purified from yam tuber [2]. As the results, we found that all chitinase isozymes do not play a role in plant defense, but only chitinase isozymes with certain properties, such as yam class IV chitinase, which belongs to family 19, do it. Just before doing this research, we assumed that since family 19 chitinases are specific to plants, family 19 chitinases would demonstrate strong lytic activity against plant pathogens, but not family 18 chitinases. However, the results showed that strong lytic activity is not specific to only family 19 chitinases but also family 18. We further investigated yam class IV on its nucleotide and amino acid sequences by cloning the genomic DNA [3]. The three-dimensional structure visualized by computer analysis suggested that yam class IV chitinase has an advantage in binding the substrate such as plant pathogen during the enzymatic action. That is the class IV chitinase is supposed to bind only four N-aceytlglucosamine moieties, whereas class I/II chitinase needs six moieties to bind the substrate with the same binding energy [3]. Therefore, we planed to use yam class IV chitinase as a defense enzyme for application to agriculture.

Secondly, in order to clarify the role of insect chitinases, we first investigated the appearance of chitinase in the silkworm during the larvae to pupae ecdysis, First the larger chitinase with 88-kDa appeared, and then 65-kDa and 54-kDa chitinases appeared in turn [4]. Furthermore, we investigated the induction of chitnases using ecdysial hormone and its inhibitor. As the results, it is proposed that these chitinase isozymes are involved in insect ecdysis. The larger chitinase would be first induced to cleave cuticle chitin in solid state, and then the smaller chitinases hydrolyzed the produced-chitooligosaccharides to the final product of N-acetylglucosamine by synergistic action together with N-acetylglcosaminidase, suggesting that the old cuticle chitin would be degraded, and

then the produced N-acetylglucosamine is reused for synthesis of new cuticle chitin. We also clarified that these smaller chitinase isozymes are in turn produced from the larger isozyme with 88-kDa, suggesting that the silkworm chitinases are produced by the post-translational processing [4]. Since these N-terminal amino acid sequences are the same, the processing to the smaller chitinases would occure by removing the C-terminal amino acid sequence. Recently we also found the 75-kDa silkworm chitinase having the same N-terminal sequence as the other chitinases [5]. Probably these 88-kDa and 75-kDa chitinases would have the additional chitin binding ability due to the small Km values, meaning that theses chitinases have chitin binding domain. Furthermore, we could demonstrate that the alternative splicing occurs in the silkworm chitinase [6]. It is the first report that enzyme isozymes are produced from one gene by both transcriptional and post-translational processings [4, 6, 7]. In this paper, we compared several chitinases on insecticidal activity against an insect pest such as Japanese pine sawyer.

Material and Methods

Chitinases Yam (*Dioscorea opposita* Thunb) class IV chitinase was purified from the tuber by DEAE-column chromatography, chromatofocusing and gelfiltration [2]. The silkworm (*Bombyx mori*) 75-kDa chitinase was purified from fifth-instar larvae by column chromatograpy on DEAE Toyopearl, hydroxylapatite, and Fractogel EMD-DEAE [5].

Construction of cDNA of chitinase A cDNA encoding yam class IV chitinase was obtained on the basis of the genomic DNA [7] by PCR, deleting the intron and vacuolar targeting signal. A cDNA encoding Japanese pine sawyer (*Monochamus alternatus* Hope (Coleoptera: Cerambycidae) larval chitinase was obtained from its mRNA by 3'RACE and 5'RACE method.

Transgenic strawberry expressing yam class IV chitinase The cDNA encoding yam class IV chitinase was introduced in strawberry by *Agrobacterium* binary system. A transgenic strawberry expressing excellent properties was selected from about 400 transformed strawberry seedlings.

Production of chitinase by Pichia pastoris *expression system* The cDNA was inserted in a plasmid of pGAPZαA or pPICZαA, and introduced into *Pichia pastoris* by electroporation. The chitinase produced in the medium of the recombinant *Pichia* was purified by DEAE-column chromatography. The yam class IV chitinase produced by the recombinant *Pichia* was treated with Endo H to remove Asn-bound saccharide.

Effect of chitinase on pathogen The strawberry seedlings (*Fragaria ananassa* Duch, Toyonoka) infected with powdery mildew (*Sphaerotheca humuli* (de Candolle) Burrill) were treated with yam class IV chitinase purified from the tuber or from the transformed *Pichia* by spraying chitinase solution (50 ml of 0.3 or 3 μM per seedling [9]. As an adhesive agent, 0.02% Kumiten (a nonionic detergent containing mainly polyoxyethylene(n)phenyl ester) was used. The evaluation was done by microscopy and scanning electron microscopy (SEM). The transformed *Pichia* produced-yam class IV chitinase was also used to investigate its effect against the powdery mildew.

Effect of chitinase on Japanese pine sawyer Japanese pine sawyer (*Monochamus alternatus* Hope (Coleoptera: Cerambycidae) adults of both sexes were obtained from a colony established at the Forestry Guidance Institute, Yamaguchi Prefecture, Japan, and maintained in gauze-covered cages

supplied with pine branches at 25°C and short day conditions (12 h light/ 12 h dark). Three hours before the treatment, the adult beetles were starved, and then orally infected with 50 μl of the silkworm 75-kDa chitinase [8], yam class IV chitinase, or *Pichia*-produced Japanese pine sawyer larval chitinase (0.3 or 3 μM in 10 mM Na-phosphate buffer (pH 8.0) containing 0.02% Kumiten) using a micro-syringe. After the injection, the beetles were supplied with two young pine branches. The mortality and the body weights of the tested beetles were examined every 6 h for a total period of 48 h. In order to investigate the effect of chitinase, peritrophic membrane in the digestive tube was obtained by dissection. The isolated peritrophic membrane was observed by both fluorescence microscopy and scanning electron microscopy (SEM). To measure the chitin content, the peritrophic membrane was observed with a fluorescence microscope by excitaion with 420 nm light after staining with 0.01% chitin-binding fluorescent dye (Fluostain®III, Dojin Chemical Laboratories, Tokyo) dissolved in 100 mM Tris-HCl buffer (pH 9.0). For SEM, the specimens were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M Na-phosphate buffer (pH 7.2) for 24 h at 4°C, and then post-fixed in 1% (w/v) OsO_4 dissolved in the same Na-phosphate buffer for 2 h at 4°C in the dark. After washing with the same Na-phosphate buffer, specimens were dehydrated in a series of ethanol solutions (50-100%) . They were then coated with 20 nm gold, and observed by SEM.

Results and Discussion

Charateristics of yam class IV chitinase Yam class IV (family 19) chitnase, which was previously named chitinase E, was induced from yam callus by inoculation with either alive and autoclaved Fusarium, chitin and chitooligosaccharides, and ethylene. It has strong lytic activity against *Fusarium* species [1]. The molecular mass is 33.5 kDa, The pI value is 3.8, meaning an acidic protein. N-terminal amino acid is glutamine, but it is pyroglutaminated. The optimum pH is 4.0 and 8.0 toward glycolchitin, and 3.0 towarad N-acetylchitopentaose. The optimum temperature is 70^{o}C. It is stable between pH 5-11, and below 70 ^{o}C. The kinetic analysis showed that it is an endo- or random-type chitinolytic enzyme catalyzing in the inverting mechanism. The kcat, Km and kcat/Km values toward glycolchitin are 0.629 1/sec, 0.639 mg/ml, 0.984 ml/mg/sec, respectively, at pH 4.0, and 0.645 1/sec, 0.518 mg/ml, 1.25 ml/mg/sec, respectively, at pH 8.0. These properties are suitable for application to agriculture as a defense enzyme against pathogens [2]. Furthermore, three-dimensional structure was visualized by computer modeling using barley seed class I/II chitinase as a template. By comparison with class I/II chitinase, it is suggested that yam class IV chitinase has an advantage as follows: the catalytic domain of yam class IV chitinase is shorter than that of class I /II chitinase due to the deletion of four small regions, which are involved in binding to the substrate, chitn. Barley seed class I/II chitinase bind six N-acetylglucosamine moieties, whereas yam class IV chitinase binds four moieties and not both end-moieties, even though the binding energy is almost the same [3].

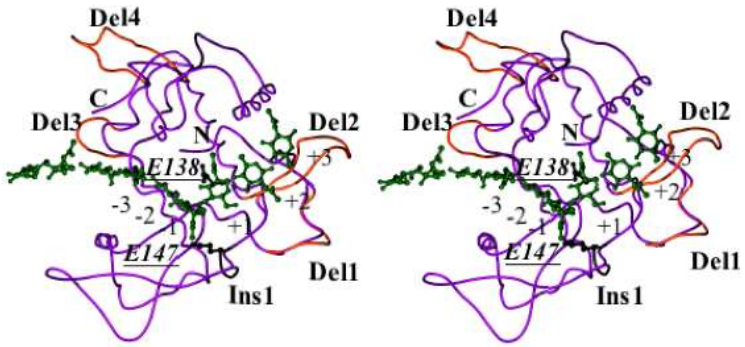

Fig. 1. Three-dimensional structure model of yam class IV chitinase.

(Fig. 1) Considering that cell walls of pathogens are composed with heterogeneous polysaccharides such chitin and glucan, the smaller recognition site is the better for a defense-enzyme of chitinase.

Application of yam class IV chitinase to development a transgenic plant resistant to pathogens We developed transgenic strawberry expressing yam class IV chitinase in collaboration with a certain Prefecture Agriculture Station. The transgenic strawberry was resistant to powdery mildew, compared with wild type strawberry.

Wild type strawberry Transgenic strawberry

Fig. 2. *Transgenic strawberry expressing yam class IV chitinase shows resistance to the powdery mildew,* Sphaerotheca macularis.

(Fig. 2) Only one excellent transgenic strawberry producing active yam chitinase was selected from about 400 transgenic strawberry seedlings. We have tried to develop many transgenic plants. Most of them kept the exogenous chitinase gene for only about three months, but later the introduced exogenous chitinase activity disappeared probably due to the silencing mechanism. In the successful case, yam class IV chitinase was attached with the signal sequence of sugar beet class IV chitinase for secretion, suggesting that it is better to use the combination of signal sequence, catalytic domain, etc, from plants different to the host plant. If we could develop lots of crops and plants resistant to pathogens, we can reduce the use of the chemicals which cause environment and health problems. In Japan, however, most of the public does not allow us to develop transgenic crops.

Application of chitinase as bio-fungicides As an alternative method to development of transgenic plants, we investigated whether yam class IV chitinase purified from the tuber can be used as a bio-control agent with a combination of β-1,3-glucanase by spraying to powdery mildew-infected strawberry. We succeeded in curing the disease and preventing the re-infection for more than 2 weeks [9].

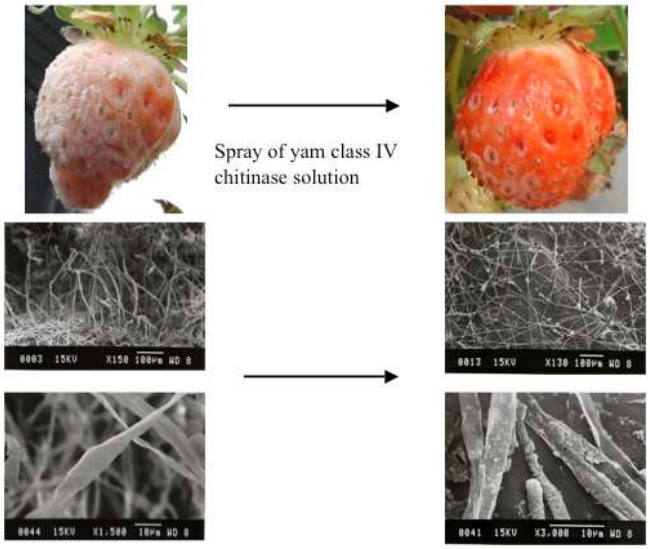

Fig. 3. Effect of yam class IV chitinase on powdery mildew infecting strawberry.

Powdery mildew-infected strawberries were treated by spraying a mixture (50 ml) of 3 μM yam class IV chitinase and 0.6% Zymolyase (β-1,3-glucanase). Before and one week later, the pathogens were observed with eyes (top) and by SEM (bottom)..

(Fig. 3) The effect was confirmed by observation by SEM. Yam class IV chitinase degraded the hyphae and conidia of powdery mildew, whereas β-1,3-glucanase could not degrade them. However, the addition of β-1,3-glucanase enhanced the lytic activity of yam class IV chitinase. These results suggest that the cell wall of strawberry powdery mildew would be constructed mainly of chitin. Next we investigated whether the yam class IV chitinase produced by the recombinant *Picha* is also effective to the powdery mildew. The yam class IV chitinase was highly glycosylated in the recombinant *Pichia* to increase in the molecular mass from 33.5 kDa to about 67-kDa, and did not indicate the lytic activity. After the treatment with Endo H, the *Pichia*-produced chitinase was deglycosylated to the original molecular mass of 33.5 kDa, and showed high lytic activity against the powdery mildew as well as the chitinase purified from yam tuber.

Application of chitinase as bio-insecticides In order to investigate the possibility of bio-inscticide, we used 75-kDa chitinase purified from the silkworm [5], yam class IV chitinase [2] and larval Japanese pine sawyer chitinase produced in the transformed *Pichia* against Japanese pine sawyer as an insect pest. Each of these chitinases was orally injected into the digestive tube of adult Japanese pine sawyer. The effect in mortality was observed in the order of silkworm 75-kDa chtinase and then yam class IV chitinase. However, *Pichia*-produced larval Japanese pine sawyer chitinase. These results were explained by the result of staining of peritrophic membrane chitin with a chitin-binding fluorescent dye.

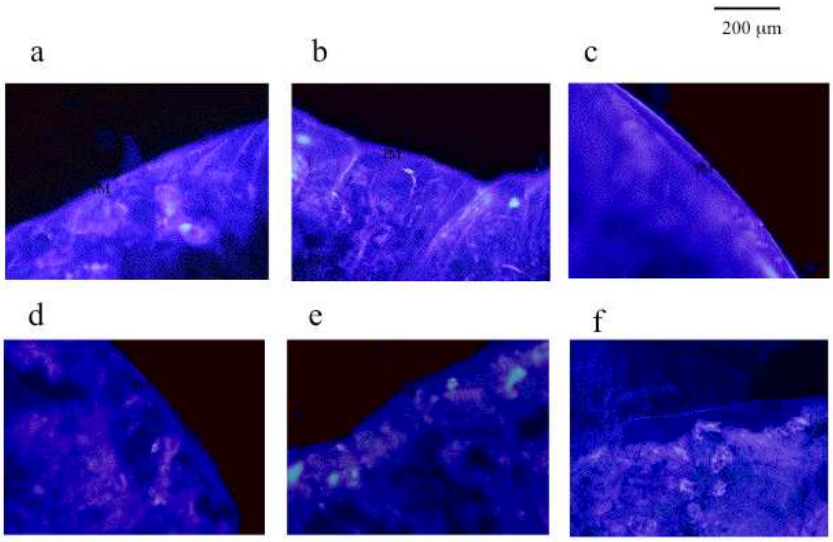

Fig. 4. Fluorescence micrographs of peritrophic membrane of Japanese pine sawyer.
Adult Japanese pine sawyer was orally ingested with 10 mM phosphate buffer (pH 8.0) (top, control) or 3 μm silkworm 75-kDa chitinase (bottom). Forty eight hours later, peritrophic membrane was dissected from the midgut region and inspected by fluorescence microscopy under UV light. Symbols: a and d, anterior mid gut; b and e, mid midgut; c and f; posterior midgut.

(Fig. 4) The silkworm 75-kDa chitinase hydrolyzed the peritorophic membrne chitin specially in the mid mdigut. In the case of silkworm 75-kDa chitinase, injection of 11.25 μg (50 μl of 3 μM) caused in 48 hr high mortality (75%), significant decrease in bark consumption (about 1/20), and slight reduction in body weight, compared with the control [8]. In order to confirm this effect, the damage of the peritrophic membrane was observed by SEM.

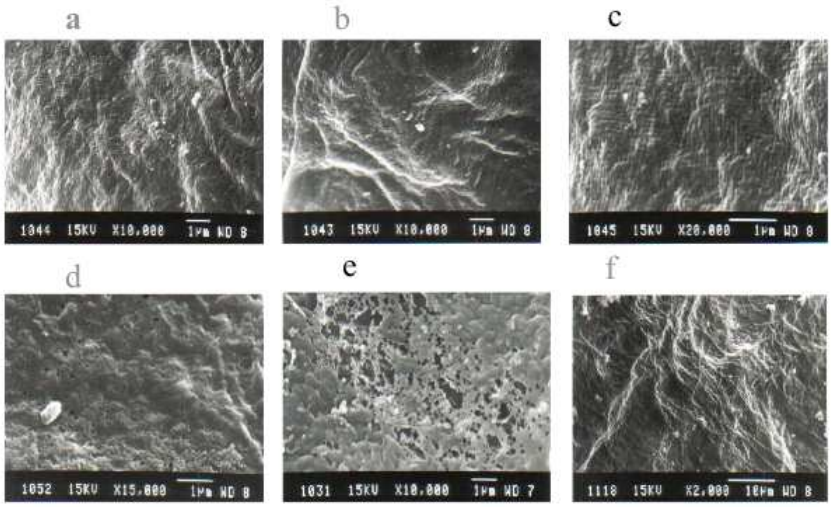

Fig. 5. Scanning electron micrographs of peritrophic membrane of Japanese pine sawyer. Adult Japanese pine sawyer was orally ingested with 10 mM phosphate buffer (pH 8.0) (top, control) or 3 μm silkworm 75-kDa chitinase (bottom). Forty eight hours later, peritrophic membrane was dissected from the midgut region and inspected by scanning electron microscopy. .Symbols: a and d, anterior mid gut; b and e, mid midgut; c and f; posterior midgut.

As shown in Fig. 5, the peritrophic membrane of the midgut was drastically degraded, specially mid midgut. These results suggest that the lining of the peitrophic membranae was composed of mainly chitin, and it is easily degraded by chitinase. Probably endogenous chitinase would control of the thickness of peritrophic membrane and the uptake or penetration of nutrient. Therefore, we can control the insect pests using exogeneous chitinase..

Evaluation of chitinase as bio-control agents We obtained fruitful results on the bio-fungicide, while only the possibility on the bio-pesticide, Since pathogens contain chitin in the cell wall, it is easy to attack the cell wall by exogenous chitinase. However, it is difficult to attack the chitin in insect cuticle, because the cuticle is covered with a wax layer and waterproof. Therefore, we changed the target from cuticle chitin to peritrophic membrane chitin. However, we have to still improve spraying method in addition to the problems in mass-production of chitinase in the recombinant microorganisms. In conclusion, we propose that the additional chitin-binding domain is necessary in the chitinase structure for development of bio-control agents, both bio-fungicide and bio-pesticide. We also proposed that plant chitinase such as yam class IV chitinase is suitable for the bio-fungicide, while insect chitinase including chitin-binding domain is suitable for the insecticide.

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