Journal of Metals, Materials and Minerals. Vol. 15 No. 1 pp. 33-36, 2005

Application of Chitinase in Agriculture

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Abstract

In order to clarify what kind of chitinase is related to plant defense system, we purified several isozymes of chitinase from yam and analyzed their physicochemical and kinetic properties and their nucleotide and amino acid sequences. Furthermore, we investigated their induction patterns and potentials as fungicide and pesticide. As the results, we found that yam (*Dioscorea opposita* Thunb) class IV (family 19) chitinase is a suitable enzyme for application to agriculture. We developed the transgenic strawberry resistant to the powdery mildew by introducing yam class IV chitinase gene. We also demonstrated that yam class IV chitinase was useful as a bio-control agent by spraying it directly to the powdery mildew (*Sphaerotheca humuli* (de Candolle) Burrill) infecting the strawberry (*Fragaria ananassa* Duch). Furthermore, we could indicate that yam class IV chitinase and the insect chitinase from the silkworm (*Bombyx mori*) had potential as insecticides against the pine sawyer beetle (*Monochamus alternatus*), which is one of the important factors causing the pine tree destruction.

Introduction

We are now faced with population explosion and environment destruction, and starvation is occurring in many regions. For production of enough food, some plant diseases and pest damages are being controlled with chemical fungicides and pesticides. Unfortunately these chemicals have sometimes caused environmental and health problems. On the other hand, plants have evolved sophisticate defense system against fungal pathogens and insect pests by producing pathogenesis-related enzymes such as chitinase. Therefore, we thought that we had better learn the naturally-acquired defense mechanism from plants and use it for the defense. First, we selected the suitable chitinase isozyme from yam by analyzing the properties including lytic activity after purification. Then we tried its application to agriculture such as development of transgenic plants resistant to fungal pathogens and development of bio-control agents instead of chemical fungicide and chemical pesticide pathogenesis-related proteins.

First we investigated the induction patterns of chitianase isozymes in yam (*Dioscorea opposita* Thunb) callus by treatment with some elicitors such as live/autoclaved *Fusarium*, ethylene, and chitin/ chitosan oligosaccharides (Koga *et al.*, 1992). Then, in order to clarify the relationship between the induction pattern and the biological role, we investigated the properties of differently-induced chitinase isozymes. So we purified the chitinase isozymes from yam tuber by ammonium sulfate fractionation, anion-exchange column chromatography, chromatofocusing and gel filtration. These chitinase isozymes were named as A, E, F, G, H1, etc, an d were analyzed on the physicochemical properties such as molecular size and pI value, antigenicity (antigen-antibody reaction), enzymatic properties such as optimum conditions and stability and kinetic behavior. Furthermore, in order to elucidate their biological roles, their lytic activities against a pathogen, *Fusarium,* were measured (Arakane *et al.*, 2000). These results are summarized in Table 1.

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Table 1. Characteristics of Yam Chitinase Isozymes

	Yam Chitinase Isozyme				
	A	E	F	G	H
Lytic Activity for *Fusarium*	ND	Strong	Strong	Weak	Strong
Molecure Weight	28,000	33,500	31,000	33,500	24,500
pI	3.6	3.8	4.0	5.5	3.6
Specific Activity (Δ 420/min/mg)	1030	14.9	4.2	5.6	42.4
Optimum pH (Glycolchitin)	4.0	4.0, 8.0	3.0, 9.0	5.0	3.0, 9.0
Optimum pH (GlcNAc4 or GlcNAc5)	4.0	3.0	4.0	4.0	4.0
Optimum Temperature (℃)	60	70	60	45	70
pH Stability	6-11	5-11	5-12	9-11	3-12
Temperature Stability (℃)	45	70	60	50	80
Antigenecity					
against anti-chitinase E	ND	+++	++	++	—
against anti-chitinase H	ND	—	—	—	+++
against anti-*Bombix mori* 88kDa chitinase	ND	—	—	—	+
Inhibition Allosamidin	Inhibited	Not inhibited	Not inhibited	Not inhibited	Inhibited
ID for Allosamidin	<55μ M (pH4.0)				1.26mM (pH4.0) 44.4μ M (pH8.0)
Elicitor		*Fusarium* chitin chitosan ethylene	*Fusarium* chitin chitosan ethylene	ethylene	chitin chitosan
Kinetics	pH4.0	pH4.0, pH8.0	pH4.0, pH8.0	pH4.0	pH4.0, pH8.0
for Glycolchitin *K* m (mg/ml)	0.096	0.639, 0.518	0.146, 0.092	2.24	0.381, 0.323
k cat (1/sec)	2.36	0.629, 0.645	0.041, 0.042	0.829	1.069, 0.591
k cat/*K* m (ml/mg/sec)	24.6	0.984, 1.25	0.278, 0.451	0.37	2.803, 1.827
Family of Glycosyl Hydrolase	ND	Family 19	Family 19	Family 19	Family 18
Classification (Class)	ND	Class IV	ND	ND	ClassIII
Anomeric form	β	α	α	α	β
N-terminal of amid acide		<QNCQCDTTIY			
Occurrence (mg/100g tuber)	0.24	4.2	2.3	1.7	1.9

ND, Not Determined

Among these isozymes, yam chitinase E has interesting properties, because it is induced by all the used elicitors such as live/autoclaved *Fusarium*, ethylene, and chitin/chitosan oligosaccharides, and also has strong lytic activity. Therefore, we concluded that this chitinase is a suitable enzyme for application to agriculture. In order to obtain the gene encoding yam chitinase E, the amino acid sequence was analyzed. Although the N-terminal amino acid was pyroglutaminated, the N-terminal amino acid sequence was successfully analyzed, suggesting that yam chitinase is classified into class IV (family 19) and have the chitin-binding domain at N-terminal region. By using the information of the amino acid sequence, the genomic DNA encoding chitinase E (class IV, family 19) was cloned. The whole amino acid sequence could be deduced (Mitsunaga *et al.*, 2004). The three-dimensional structure was also visualized by computer modeling using the x-ray structure of barley seed chitinase (class II) as a template (Mitsunaga *et al.*, 2004) (Figure 1) This three-dimensional structure suggests that chitinase E (class IV, family 19) has an advantage. That is, yam class IV chitinase recognizes an even shorter segment of the substrate such as fungal pathogen cell walls than class I or II chitinases, and can attack pathogens more easily.

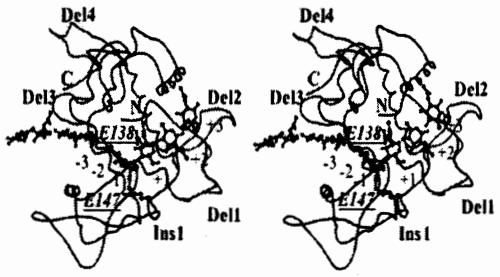
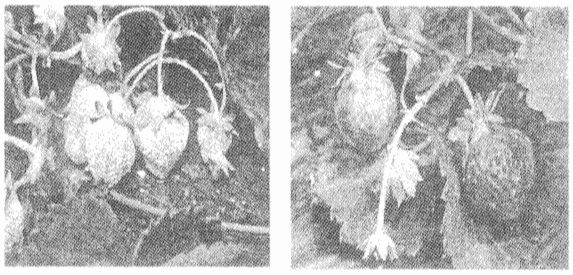


Figure 1. Three-dimensional structure model of yam class IV chitinase.

Transgenic strawberry carrying yam class IV chitinase gene

Some transgenic plants which were resistant to fungal pathogens have been developed since 1991 by using several chitinase genes. However, some of the used chitinase genes were effective for development of the transgenic plants, but not all. For example, as shown in Table 1, yam chitinase G is not suitable, because its lytic activity is not strong. Therefore, we selected the suitable chitinase isozyme, yam chitinase E (class IV, family 19), from many isozymes to develop the transgenic plants. As shown in Figure 2, the transgenic strawberry carrying its gene showed

the resistance to the powdery mildew (*Sphaerotheca humuli* (de Candolle) Burrill). In contrast, the wild type strawberry was infected severely by the powdery mildew with white powder.



Wild type strawberry Transgenic strawberry

Figure 2. Transgenic strawberry carrying yam class IV chitinase shows resistance to powdery mildew.

Yam class IV chitanase as a bio-control agent against strawberry powdery mildew

As shown in Figure. 2, we succeeded in thedevelopment of the transgenic strawberry resistant to powdery mildew. In Japan,howeve r, transgenic crops are not acceptedby the publi c. Therefore, we have to find outalternative me thods. Since we had the datathat yamchitinase E(classIV, family19) is very stable even under high temperature, we thought thatit is possible to use it as a bio-control agent by spraying in the field. Furthermore,since we succeeded in t he developmentof the transgenic strawberry resi stant to the powdery mildew, yam chitinase E (class IV, family 19) wouldbe effective against the powdery mildew even in the field. When yam chitinase E solution (0.3 to 3 μM) was sp rayed to strawberry powdery mildew (*Sphaeroth ecahumuli)* infecting the leaves and berries of t he strawberry plant, most of the white powder on the infected part of the berries disappeared immediately. One week later the disease had m icroscopy (SEM) showed that the hyphae of th e powdery mildew were severely damaged, wit h holes in the surface (Karasuda *et al.*, 2003) Figure 3. Infection of the leaves stopped sprea ding, and the infected spots gradually turned fr om white to brown. This effectwas enhanced b y the additional β-1,3-glucanase. We have alrea dy produced a large amount of yam chitinase E in the *Pichia* system,that was also effective against the powdery mildew as well as naturall y-occurring yam chitinase E.

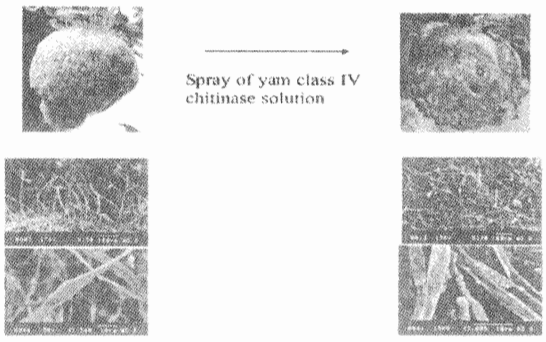


Figure 3. Effect of yam class IV chitinase on Powdery mildew as a biocontrol agent.

Chitinase as a bio-control agent against the pine sawyer beetle

In order to indicate the possibility of use of chitinase as a bio-control agent against insect pests, we tried to use both yam chitinases (50 μ liter, 0.3 to 3 μM) were individually administrated orally into the digestive tube of the pine sawyer beetle (*Monochamus alternatus*), which is one of the important factors causing the pine tree destruction in Japan, most of the beetles died in 20 hrs. The damage of peritrophic membrane was also observed with a fluorescent microscope and SEM, suggesting that both chitinases have potential as a pest-control agent (bio-control agent).

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