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Biodegradation of Cry1Ab Protein from Bt Transgenic Rice in Aerobic and Flooded Paddy Soils

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Degradation of Cry1Ab protein from Bt transgenic rice was examined under both aerobic and flooded conditions in five paddy soils and in aqueous solutions. The hydrolysis rate of Cry1Ab protein in aqueous solutions was correlated inversely with the solution pH in the range of 4.0 to 8.0, and positively with the initial concentration of Cry1Ab protein. Rapid degradation of Cry1Ab protein occurred in paddy soils under aerobic conditions, with half-lives ranging from 19.6 to 41.3 d. The degradation was mostly biotic and not related to any specific soil property. Degradation of the Cry1Ab protein was significantly prolonged under flooded conditions compared with aerobic conditions, with half-lives extended to 45.9 to 141 d. These results suggest that the toxin protein, when introduced into a paddy field upon harvest, will probably undergo rapid removal after the field is drained and exposed to aerobic conditions.

KEYWORDS: Cry1Ab protein; degradation; Bt transgenic rice; paddy soil

INTRODUCTION

In recent years, genetically modified (GM) crops have been adopted in 21 countries covering more than 90 million hectares and with an annual rate of increase of over 10%. As many as 20 million hectares are occupied by insect-resistant transgenic plants (*1*). *Bacillus thuringiensis* (Bt) is the major source of genes for the expression of pest resistance in transgenic plants. Truncated forms of the genes that code for Bt toxin protein have been genetically engineered into plants. However, the ecological effects of GM crops remain controversial and poorly understood. Transgenic rice with insect resistance may soon become the first approved major food crop in China. During the growth of Bt transgenic plant, expressed toxin protein may enter the environment via discarded biomaterials or straw returned-to-soil practices (*2*,*3*), which has drawn wide attention due to the potential environmental implications (*4*−*8*). Many studies have reported the environmental behavior and fate of the insecticidal toxins (*9*−*17*). Venkateswerlu and Stotzky (*18*) studied binding of the protoxin and toxin protein of *B. thuringiensis* subsp. *kurstaki* on clay minerals and found that the toxin was readily adsorbed on clay minerals. Stotzky (*16*, *17*) investigated the persistence and biological activity of insecticidal proteins from *B. thuringiensis* in soil and binding of bacterial DNA on clays and humic acids. Crecchio and Stotzky (*10*) reported biodegradation and insecticidal activity of the toxin from *B. thuringiensis* subsp. *kurstaki* bound on complexes of montmorillonite-humic acids-Al hydroxypolymers. Zhou et al. (*19*) demonstrated adsorption of the insecticidal protein of *B. thuringiensis* on montmorillonite, kaolinite, silica, goethite, and Red soil. Most of these studies used plant materials or Cry1Ab protein produced by *B. thuringiensis*, instead of free protein extracted or purified from Bt transgenic plants. However, studies using free Cry1Ab protein produced by Bt transgenic plants may provide protein-specific information on its environmental behavior and fate.

Rice paddy soils are anaerobic during the period of plant growth when the field is flooded and aerobic during the period between the harvest of the mature crop and the following planting of rice seedlings. In addition, succeeding crops after rice harvest are often wheat, canola, and other upland crops in the south of China. Thus, toxin proteins from transgenic plants may be exposed to both aerobic and anaerobic soil conditions, and it is essential to understand their persistence under aerobic and flooded conditions. Tapp and Stotzky studied the persistence of the insecticidal toxin from *B. thuringiensis* subsp. *kurstaki* in soil (*14*). However, there have been no comparative studies on the degradation and stability of Bt rice toxin proteins in paddy soils under aerobic and anaerobic conditions.

The objective of this study was to evaluate the influence of aerobic and flooded conditions on the degradation of purified Cry1Ab protein from Bt transgenic rice in paddy soils. The stability of the protein in aqueous solutions, as influenced by pH and initial protein concentration, was also characterized under laboratory conditions.

MATERIALS AND METHODS

Rice Straw and Soils. The Bt transgenic rice used was the transgenic rice line Ke-Ming-Dao (KMD) in the TR_{12} generation with a homozygous *cry1Ab* gene. This cultivar was developed from a commercial

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Table 1. Some Basic Physicochemical Properties of the Five Paddy Soils Used

soil no.	type	pH (H_2O)	OM (g kg^{-1})	CEC (cmol kg^{-1})	clay (%)	silt (%)	sand (%)	total N (%)
S_1	paddy field on quaternary red soil	4.16	8.40	6.62	39.0	41.1	19.9	0.34
S_2	paddy field on red sandstone soil	4.55	6.53	4.53	17.2	7.4	75.4	0.28
S_3	fluvio-marine yellow loamy soil	7.02	30.50	10.83	8.0	71.2	20.8	2.90
S_4	coastal saline soil	8.84	9.50	10.17	24.3	71.1	4.6	1.80
S_5	paddy field on pale muddy soil	5.81	25.11	8.15	49.3	11.4	39.3	2.70

[a] Note: OM, organic matter; CEC, cation exchange capacity.

Chinese *Oryza japonica* rice variety Xiushui 11 and transformed by the *Agrobacterium*-mediated method (*20*). The transgenic rice line KMD contains a synthetic *cry1Ab* gene from *B. thuringiensis* subsp. *kurstaki*, under the control of a maize ubiquitin promoter and linked in tandem with the *gusA*, *hpt*, and *npt II* genes (*21*,*22*). The plants were resistant to eight lepidopteran rice pest species when subjected to either artificial or spontaneous attacks (*23*).

The five soils used in the incubation experiments were collected from the surface layer (0−15 cm) in fields from parts of Zhejiang province, where no transgenic rice had ever been planted. The soils were air-dried, mixed, passed through a 1-mm sieve, and homogenized in a rotary cylinder before use. Some basic physicochemical characteristics of these soils are shown in **Table 1**.

Preparation of Purified Cry1Ab Protein. Straws of the Bt transgenic rice KMD and nontransgenic rice (the wild type Xiushui 11) were harvested at the booting stage when the content of expressed Bt toxin proteins in KMD was expected to reach its highest level. KMD straw was treated with liquid nitrogen and ground in an Udy cyclone mill (0.3 mm; Udy Corporation, Fort Collins, CO), and the resulting straw powder was stored at 4 °C before use. Cry1Ab protein from Bt transgenic rice was purified as described by Wu and Ye (*24*) and determined by the method of Wang et al. (*25*). Crude extracts were obtained after pretreatment with freeze-drying concentration, ammonium sulfate precipitation, desalinization, ultrafiltration, and freeze-drying concentration again. The dialyzed crude protein was further separated on DEAE Sephadex A-50 columns and Sephadex G-150 columns. Each purification step contributed to the improvement of Cry1Ab protein purity, which was determined by SDS-PAGE. In order to get desirable purity, the ion-exchange chromatographic separation can be performed once. Ultimately, the purity was obtained higher than 80%. All steps were performed at 4 °C.

Hydrolysis Experiment. Hydrolysis of purified Cry1Ab protein was determined in aqueous solution with different pH. The purified toxin protein was dissolved in 0.1 M phosphate buffer (pH 6.0). The pH was slowly brought to 4.0, 5.0, 7.0, and 8.0 with phosphate buffer. The final concentration of protein in the solutions at different pH was 500 ng mL^{-1} as determined by ELISA (*25*). Ten mL of each solution was transferred into screw-cap glass test tubes and incubated at 25 ± 1 °C in the dark. Meanwhile, hydrolysis of purified Cry1Ab protein as a function of the initial protein concentration was also measured at pH 7.0. Sample tubes with 10 mL of solutions (pH 7.0) containing the Cry1Ab protein at 300, 500, 1000, 2000, and 4000 ng mL^{-1} were prepared. Three replicates of each treatment were sealed and incubated at 25 ± 1 °C in the dark. After different intervals of incubation, 100 μL of the solution was withdrawn with pipettes (Eppendorf AG, Hamburg, Germany), and the concentration of Cry1Ab protein was determined by ELISA using a Quantiplate kit (AP 003, Envirologix Co., Porland, ME) at 450 nm in a SpectraMax 190 Spectrophotometer using Softmax software (Molecular Devices, Sunnyvale, CA).

Soil Incubation Experiments. Soil incubation experiments were conducted under controlled conditions to determine the persistence of Cry1Ab protein in aerobic and flooded paddy soils. In the aerobic degradation experiment, 50 mL of purified Cry1Ab protein was thoroughly mixed with 50 g of soil (S_1 to S_5) in 250-mL flasks to achieve an initial concentration of 560 ng g^{-1} air-dried soil. The water content of the spiked soil samples was adjusted to 60% of the water-holding capacity. All treatments were prepared in triplicate. The soil flasks were loosely covered with rubber stoppers and incubated at 25 ± 1°C in the dark. During the incubation, the flasks were opened and aerated daily for 0.5 h, and the water content was maintained by adding distilled water when necessary. After different intervals of incubation (0, 3, 5, 10, 15, 20, 30, 60, 90, 120, and 150 d), a 0.5-g (air-dried weight equivalent) subsample of the soil was removed from each flask and extracted using the procedures described by Wang et al. (*25*). Each of the 0.5-g soil samples were mixed with 1.0 mL extraction buffer (0.1 M Na_2CO_3 + 0.1 M $NaHCO_3$ + 5 mM EDTA + 10 mM DTT + 50 mM $Na_4P_2O_7 \cdot 10H_2O$ + 0.1% Triton X-100, pH 10) on a shaker for 1 h, followed by centrifugation at 12 000 rpm for 10 min. The remaining soil was consecutively extracted for two additional times. The resulting supernatants were combined, and determination of Cry1Ab protein was performed by ELISA.

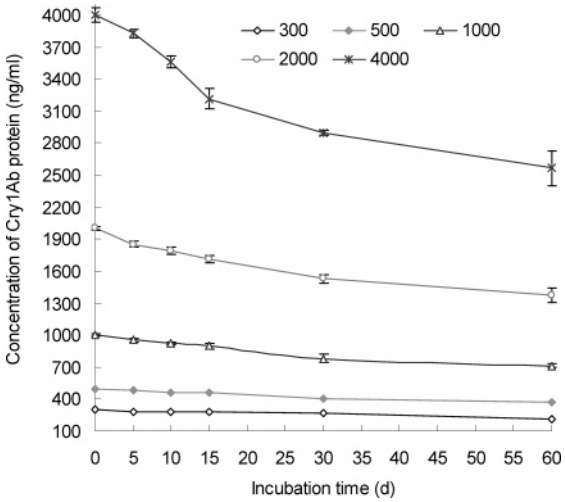

Figure 1. Hydrolysis of purified Cry1Ab protein in aqueous solutions (pH 7.0) at different initial protein concentrations (ng mL^{-1}). Each point is the mean of three replicates, and the bars represent standard errors.

An anaerobic incubation experiment was performed using three soils (S_1 to S_3). The soils (50 g) in 250-mL flasks were amended with 50 mL of purified Cry1Ab protein to 560 ng g^{-1} air-dried soil. The soil was flooded with distilled water to the depth of 2 cm, and the flasks were closed tightly with rubber stoppers. All soil samples were prepared in triplicate and incubated at 28 ± 1°C in the dark without mixing. After different intervals of incubation, 100 μL of aqueous phase and 0.5 g (air-dried weight equivalent) of soil were removed from each flask. The protein content of the liquid phase was directly determined by ELISA. The protein content in soil phase was determined following the same procedures as described above.

Statistical Analysis. All data are expressed as geometric mean values and standard errors of the means (mean ± SEM). Significance between treatments was determined by Student's *t*-test, and a one-way analysis of variance (ANOVA) was done for regression analysis using Origin 6.0 (Microcal Software, Northampton, MA).

RESULTS AND DISCUSSION

Hydrolysis of Purified Cry1Ab Protein in Aqueous Solution. Figure 1 shows the hydrolysis of the Cry1Ab protein in neutral aqueous solution (pH 7.0) at initial protein concentrations ranging from 300 to 4000 ng mL^{-1}. Hydrolysis of the Cry1Ab protein at each of the five concentrations during 60 d of incubation was well described by the first-order kinetics model

Table 2. First-Order Regression Analysis of the Hydrolysis of Cry1Ab Protein in Solution of pH 7.0 as a Function of Initial Protein Concentration

concn (ng/mL)	rate constant k(d^{-1})	half-life $t_{1/2}$ (d)	correlation coefficient (r)	p
300	0.0053	130.8	0.98	0.001
500	0.0054	128.4	0.98	0.001
1000	0.0057	121.6	0.98	0.001
2000	0.0060	115.5	0.97	0.001
4000	0.0074	93.7	0.96	0.03

Table 3. Regression Analysis and Half-Life Values of the Hydrolysis of 500 ng mL^{-1} of Cry1Ab Protein in Aqueous Solutions at Different pH

pH	rate constant k (d^{-1})	half-life $t_{1/2}$ (d)	correlation coefficient (r)	p
4.0	0.0084	82.5	0.99	0.000
5.0	0.0069	100.4	0.93	0.007
6.0	0.0062	111.8	0.99	0.000
7.0	0.0054	128.4	0.98	0.001
8.0	0.0033	210.0	0.95	0.004

Table 4. First-Order Regression Analysis of the Degradation of Cry1Ab Protein in Paddy Soils under Aerobic and Anaerobic Conditions

soil no.	rate constant k (d^{-1})	half-life $t_{1/2}$ (d)	correlation coefficient (r)	p
		Aerobic		
S_1	0.0262	26.5	0.99	<0.005
S_2	0.0168	41.3	0.96	<0.005
S_3	0.0180	38.5	0.96	<0.005
S_4	0.0353	19.6	0.97	<0.005
S_5	0.0293	23.7	0.97	<0.005
		Anaerobic		
S_1	0.0049	141.4	0.99	<0.005
S_2	0.005	138.6	0.90	<0.05
S_3	0.0174	45.9	0.94	<0.005

$C = C_0 \exp(-kt)$, with r ranging from 0.96 to 0.98 ($p < 0.03$, **Table 2**). The half-life estimated for the persistence of the Cry1Ab protein varied from 93.7 to 130.8 d. The shortest half-life (93.7 d) was in the solution containing the protein at the highest concentration (4000 ng mL^{-1}); at the end of the 60-d incubation, 32.5% of the initially added Cry1Ab had disappeared from the solution. In contrast, in the solution with a low initial protein concentration (300 ng mL^{-1}), the half-life increased to 130.8 d. These results suggest that the Cry1Ab protein from transgenic rice straw was relatively resistant to hydrolysis at neutral pH, and therefore abiotic degradation may contribute insignificantly to the dissipation of Cry1Ab protein in paddy soils. In addition, the results suggest that the stability of Cry1Ab protein in neutral aqueous solutions may be negatively related with the initial protein concentration, and a shorter persistence may be expected at high protein concentrations.

The disappearance of Cry1Ab protein in buffer solutions with different pH also followed the first-order decay model, with r ranging from 0.93 to 0.99 ($p < 0.01$) (**Figure 2**; **Table 3**). The half-lives of Cry1Ab estimated from the first-order decay regression increased from 82.5 to 210 d as the solution pH increased from pH 4.0 to pH 8.0. At the end of the 60-d incubation, only about 16% of the initially added Cry1Ab protein was unaccounted for in the pH 8.0 solution, while as much as 38% of the added protein was lost in the pH 4.0 solution. Regression between pH and the hydrolysis rate constant, k,

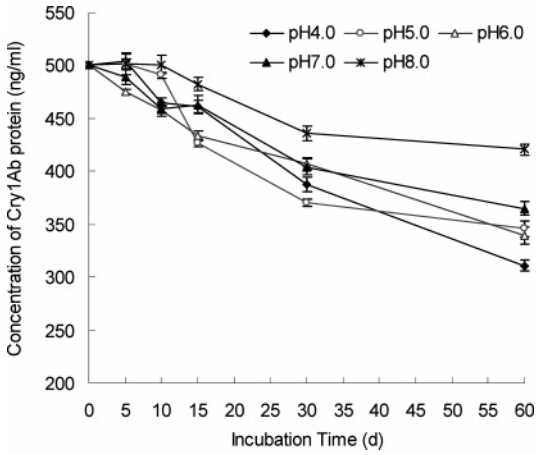

Figure 2. Hydrolysis of purified Cry1Ab protein in aqueous solutions at different pH. Each point is the mean of three replicates, and the bars represent standard errors.

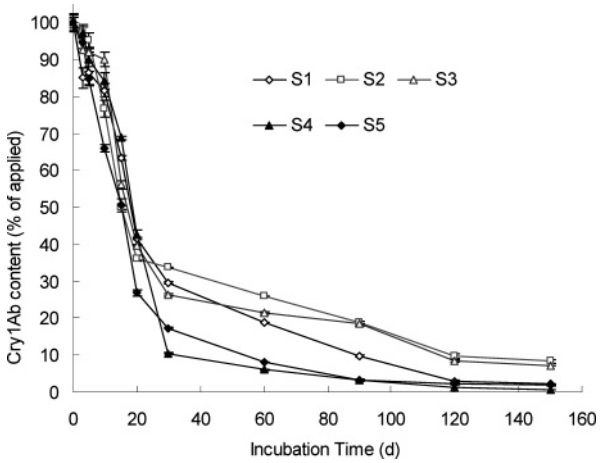

Figure 3. Degradation of purified Cry1Ab protein in paddy soils under unsaturated, aerobic conditions. Each point is the mean of three replicates, and the bars represent standard errors.

showed a negative linear correlation ($r = 0.98$) with a slope of −0.0012. The increased degradation of Cry1Ab at low pH may contribute to its shorter persistence in acidic soils that are commonly found in the rice-producing regions in southeastern China and some other Asian countries.

Degradation of Cry1Ab Protein in Paddy Soils under Aerobic Conditions. Aerobic degradation of Cry1Ab protein in the five soils during 150 d of incubation generally followed the first-order decay model, with r ranging from 0.96 to 99 ($p < 0.005$) (**Table 4**). The first-order half-lives for the extractable Cry1Ab protein varied from 19.6 to 41.3 d. The Cry1Ab protein dissipated fastest in the alkaline S_4 soil, with a half-life of only 19.6 d. Conversely, the protein was degraded substantially slower in the acidic or neutral soils, with half-life of 41.3 d for S_2 and 38.5 d for S_3. An initial rapid decline in the extractable Cry1Ab protein concentration was observed for all soils during the first 20 d of incubation, when the level of Cry1Ab protein decreased to about 25% of the initial values in S_5 and 50% in the other soils. After 30 d of incubation, the level of Cry1Ab protein in S_5 decreased to less than 10% of the initial value, and then was followed by a slower decline (**Figure** 3). After incubation for 150 d, the Cry1Ab protein was still detected in all of the 5 soils, at 8.9 ng g^{-1} air-dried soil in S_4, but at 100 ng g^{-1} or more in the other soils. Further regression analysis between the first-order rate constant, k

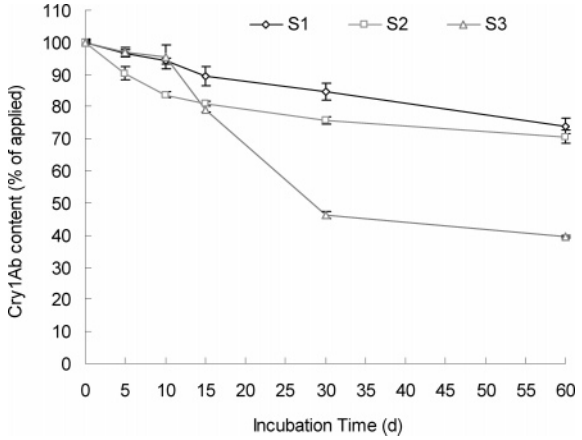

Figure 4. Degradation of purified Cry1Ab protein in paddy soils under flooded, anaerobic conditions. Each point is the mean of three replicates, and the bars represent standard errors.

(**Table 4**), and the selected soil properties (**Table 1**) did not reveal any statistically significant correlation with any of the soil properties. The variation in the persistence of Cry1Ab protein among the different soils was not directly related to soil pH, which was in contrast with the above observed dependence of the persistence of Cry1Ab protein on pH in solution. For instance, the alkaline S_4 soil had the highest pH (8.84) but the shortest half-life (19.6 d). Therefore, factors other than the soil pH may have contributed to the degradation of the Cry1Ab protein in the paddy soils. This observation also suggests that soil microorganisms probably contributed to the dissipation of Cry1Ab protein in the paddy soils and that in the alkaline S_4 soil, there was potentially enhanced biotransformation by the indigenous microbial communities. From this and other studies, degradation of Cry1Ab protein in paddy soils is largely biotic and may depend on the clay mineral composition and pH of the soils, and incubation conditions (*9*, *14*, *26*, *27*).

Several researchers have reported that the degradation of the Bt toxins does not follow a first-order kinetics. Sims and Holden (*28*) showed that degradation of Bt toxin could not be fully described by a simple first-order kinetics. Data on the degradation of the Cry3Bb1 protein expressed by Bt corn transformed by event (MON 863) also showed a nonlinear degradation kinetics (*29*). In contrast, in our study, degradation of Cry1Ab protein closely followed a first-order kinetics equation within incubation time. The difference between this and other studies may be attributed to the difference in the origin, type, and amount of protein, the method used to quantify the toxin concentration, or laboratory incubation conditions (*14*, *25*, *28*).

Wang et al. (*23*). showed that the half-life of the Cry1Ab protein in KMD straw ranged from 11.5 to 34.3 d in the same five soils amended with KMD straw (4%, wt wt^{-1}). Degradation of purified Cry1Ab protein in the same soils was slower than that in KMD straw under comparable conditions. The purified Cry1Ab protein persisted in the soils for 345 d in this study, whereas the Cry1Ab protein from Bt rice straw persisted in the soils only for 146 d (*25*). Palm et al. (*15*) also found that purified Bt protein was more stable in the soil than the toxin produced from transgenic cotton leaves. The difference happened probably because purified protein bound on surface-active particles in soil, which protected the protein against biodegradation. The similar conclusion was also found by Tapp and Stotsky (*14*).

Degradation of Cry1Ab Protein in Paddy Soils under Flooded Conditions. Degradation of Cry1Ab protein from transgenic plants in paddy soils under flooded conditions has not been evaluated previously. In this study, anaerobic degradation of Cry1Ab protein was determined in three paddy soils (S_1, S_2, and S_3) under flooded conditions. Degradation of the Cry1Ab protein was slow in S_1 and S_2 and was faster in S_3 following a 10-d lag period (**Figure 4**). In S_1 and S_2, as much as 80% of the initially added Cry1Ab protein remained in the soil at the end of the 60-d incubation. The level of Cry1Ab protein in S_3 decreased rapidly from about 95% on day 10 to 40% on day 60. Under aerobic conditions, the level of Cry1Ab protein in S_1 to S_3 decreased to 20 to 30% of the initial level after 60 d of incubation. The first-order half-lives of the Cry1Ab protein under anaerobic conditions were estimated to be 141 d for S_1, 138 d for S_2, and 45.9 d for S_3. These half-lives were considerably longer than those observed for the same soils under aerobic conditions, especially for S_1 and S_2 (**Table 4**). These results suggest that the Cry1Ab protein may be degraded in paddy soils mostly during the nonflooded periods, such as after the harvesting of the mature crop. However, the protein may be relatively stable in the paddy soils during the growing season when the soil is flooded and dominated by anaerobic conditions.

Analysis of the supernatant for the Cry1Ab protein did not show any detectable level of the protein in the solution phase. This observation suggests that once the protein was added to the flooded paddy soils, it was tightly adsorbed to the soils, and the strong adsorption may have protected the protein from biotic degradation and, thus, extended its persistence. Similar results were also reported by Stotzky (*16*,*17*), Venkateswerlu and Stotzky (*18*), and Saxena and Stotzky (*30*). Comparison of anaerobic degradation and hydrolysis showed that the degradation of Cry1Ab protein in acidic soils S_1 (pH 4.15) and S_2 (pH 4.55) under anaerobic conditions was much faster than its hydrolysis in solutions with similar pH. In the acidic soils, the Cry1Ab protein (pI 5.5) became increasingly protonated, and the positive charge may have resulted in enhanced adsorption of the protein to the net negatively charged soil humic substances and clay minerals (*11*). The enhanced adsorption may have contributed to the slower degradation of the Cry1Ab protein in the acidic soils. Tapp and Stotzky (*14*) studied persistence of the toxin from *B. thuringiensis* subsp. *kurstaki* and found that more of the toxins had degraded by microbes in soils with the higher pH values, which was exactly consistent with our results.

In conclusion, this study showed that in aqueous solutions, the rate of hydrolysis of the Cry1Ab protein increased with decreasing pH, and the protein became more stable at high pH. In a neutral pH range, the hydrolysis of Cry1Ab was dependent on the solution concentration of the protein, and faster hydrolysis occurred at higher protein concentrations. In paddy soils, degradation of the Cry1Ab protein was not correlated with any specific soil property, but the degradation was predominantly biotic in nature and was apparently influenced by adsorption of the protein to soil particles. Degradation of the Cry1Ab protein was considerably more rapid under aerobic conditions than under flooded conditions. Therefore, dissipation of the Cry1Ab protein from transgenic rice under field conditions may occur mostly after the harvest of the grains when the soil is drained, and minimal removal of the protein may be expected during the growing season when the soil is constantly flooded. The significance of our findings is important when evaluating the potential risks associated with the release to the environment of transgenic plants containing the *cry1Ab* gene.

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