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ARTICLE

Pepsin Degradation of Cry1A(b) Protein Purified from Genetically Modified Maize (*Zea mays*)

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The aim of this work was to study the *in vitro* digestion of Cry1A(b) protein by pepsin. To perform this work, a protein fraction purified from transgenic maize by immunoadsorption was employed. The undigested fraction showed several bands of molecular weight ranging between 14 and 70 kDa when assayed by SDS−PAGE. These bands were identified as corresponding to Cry1A(b) protein by immunochemical techniques and mass spectrometry. The rate of degradation of the purified fraction by pepsin estimated by ELISA was found to be about 75% within 30 min, and the protein concentration remained constant up to 4 h. In all treated samples, the full-length protein and fragments present in Cry1A(b) fraction were absent and peptides of less than 8.5 kDa were mainly found by SDS−PAGE and mass spectrometry. These peptides did not react with antiserum against Cry1A(b) protein by Western blotting. These results suggest that Cry1A(b) fraction purified from transgenic maize is rapidly and extensively degraded by pepsin, giving peptides of low molecular mass.

KEYWORDS: Cry1A(b) protein; transgenic maize (*Zea mays*); pepsin degradation; immunochemical techniques; mass spectrometry

INTRODUCTION

Cultivation of transgenic maize has increased worldwide from 0.3 million hectares (ha) in 1996 to 37.3 million ha (about 30% of global biotech area) in 2008, the dominant trait being insect resistance followed by herbicide tolerance trait and stacked genes for the two traits. In Europe, maize is the main transgenic crop, grown on over 120,000 ha (*1*). The transgenic maize lines resistant to the European corn borer express the δ-endotoxin genes of *Bacillus thuringiensis*, and so they are called Bt maize (*2*). These genes cause the production of specific insecticidal proteins known as Cry proteins, of which the Cry1A(b) protein is the most commonly expressed (*3*).

This transgenic maize is increasingly being used not only as a feed source for farm animals but also for human consumption (*4*). However, the public has shown concerns about the effect and the digestive fate of inserted DNA and expressed proteins in transgenic maize (*5*). Therefore, the biosafety aspects, regulations and labeling of foods derived from genetically modified organisms are contentious issues in many countries.

Studies conducted with animals fed with transgenic maize have shown that Cry1A(b) protein is easily degraded throughout the digestive tract, although there is a wide variability in the degree of degradation and the profile of peptides obtained in the different works (*6*−*9*).

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Studies performed with pigs and calves fed with Bt11 transgenic corn showed that more than 90% of the ingested Cry1A(b) protein was already degraded in the stomach, and only trace amounts of protein survived passage through the intestine. Using Western blotting, a band of 65 kDa mass, derived from the 130 kDa protein expressed, was detected in gastrointestinal contents and feces, but the reaction with antibodies was very weak (*6*, *7*). Likewise, experiments performed with cows fed with Bt176 maize revealed a significant degradation of the Cry1A(b) protein in the bovine gastrointestinal tract (*5*, *8*, *9*). However, in all gastrointestinal samples analyzed in these studies, only fragments of approximately 34 and 17 kDa, derived from the 60 kDa protein expressed, were found. Discrepancies between the findings of different studies could be due to differences in the metabolism of calves, pigs and lactating cows or to the different fragmentation pattern of Bt176 compared to Bt11 corn (*5*).

Some authors have studied the extent of Cry1A(b) protein degradation, using *in situ* experiments with ruminally cannulated cows. Using this technique, Jacobs et al. (*10*) determined that the amount of Cry1A(b) protein remaining after 16 and 24 h of ruminal degradation was 85% and 60%, respectively, indicating that a significant fraction of Cry1A(b) protein was not digested in the rumen. However, Wiedemann et al. (*5*) observed a higher degradation of Cry1A(b) protein in the rumen, as a decrease to 28% of the initial value was measured within 2 h, and to 2.6% within 48 h. The full-size protein was detected up to 8 h, and thereafter, only fragments of approximately 17 and 34 kDa were found.

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On the other hand, the *in vitro* degradation of Cry1A(b) protein in the presence of simulated gastric or intestinal fluids, containing pepsin or trypsin, respectively, was studied (*11*−*13*). These studies were performed using crude extracts of transgenic maize or Cry1A(b) protein purified from *Escherichia coli* transformed with Cry1A(b) gene, because an insufficient amount of this protein could be purified from plant tissue. The amount of Cry1A(b) protein was determined exclusively by Western blotting analysis using monoclonal or polyclonal antibodies. Results obtained by these authors indicated that most of Cry1A(b) protein is rapidly degraded by pepsin within 2 min or less (*11*−*13*), whereas no degradation by trypsin was observed after 4 h (*11*) or 19 h of incubation (*12*).

In the present work, the *in vitro* degradation of Cry1A(b) by pepsin was studied using electrophoresis, mass spectrometry and immunochemical techniques. To perform this study, Cry1A(b) protein was previously purified from transgenic maize using an immunosorbent containing specific antibodies against Cry1A(b) protein. To our knowledge, this is the first work on proteolysis carried out with Cry1A(b) protein purified from transgenic maize.

MATERIALS AND METHODS

Isolation and Conjugation of Anti-Cry1A(b) Antibodies. Antisera to purified trypsin-activated Cry1A(b) protein from *B. thuringiensis* were obtained in White New Zealand rabbits, and anti-Cry1A(b) antibodies purified using an immunosorbent of Cry1A(b) protein insolubilized in Sepharose 4B as previously described (*14*). Anti-Cry1A(b) antibodies were conjugated with horseradish peroxidase (HRP, 250−503 units/mg) (Sigma, Poole, U.K.) using the periodate method (*15*). The conjugate obtained was dialyzed against 0.15 M NaCl, 10 mM potassium phosphate buffer, pH 7.4 at 4 °C overnight and stored at −20 °C until use.

Purification of Cry1A(b) Protein from Transgenic Maize Leaves. Fresh leaves from transgenic maize (DKC6575 variety) containing the event MON810 were ground, and 1 kg of the ground leaves was mixed with 5 L of 0.15 M NaCl, 0.1 M sodium carbonate buffer, pH 9.5. After stirring for 2 h, the mixture was filtered through glass wool and the filtrate centrifuged at 2700*g* for 15 min. The supernatant was filtered using a 1,000 kDa Hollow Fiber Cartridge (Amicon, Danvers, MA) to eliminate particulate material, and then concentrated using a 10 kDa Pellicon 2 "cassette" filter (Millipore, Billerica, MA).

Cry1A(b) protein was purified using an immunosorbent of anti-Cry1A-(b) antibodies insolubilized in Sepharose 4B previously prepared (*14*). A volume of 10 mL of concentrate maize extract was applied to the immunosorbent, and the column was washed with 0.15 M NaCl, 10 mM potassium phosphate buffer, pH 7.4. Retained protein was eluted with 0.5 M NaCl, 0.1 M glycine-HCl buffer, pH 2.6 containing 10% dioxane and immediately neutralized with 0.5 M Tris buffer, pH 8.0. The protein was dialyzed against distilled water and the concentration determined by BCA Protein Assay (Pierce, Rockford, IL).

Pepsin Hydrolysis. An amount of 1 mg of Cry1A(b) protein from transgenic maize or from *B. thuringiensis*, dissolved in 100 μL of 84 mM HCl, pH 2.0 buffer, was hydrolyzed with porcine pepsin (4.23 units/mg) (Sigma, Poole, U.K.) with an enzyme/substrate ratio (E/S) of 5%. Proteolysis was performed at stabilized temperature of 37 °C. Aliquots were removed after 30 min, 2 h and 4 h, and hydrolysis was stopped in three different ways. Samples to be subjected to SDS−PAGE were mixed with sample electrophoresis buffer, and immediately boiled for 5 min. Samples to be analyzed by ELISA were diluted 1:200 in 8 mM Na_2HPO_4, 3 mM KCl, 0.14 M NaCl, 1.5 mM KH_2PO_4 buffer, pH 7.4 (PBS), and those to be analyzed by mass spectrometry were diluted 1:2 in ammonium carbonate buffer 50 mM, pH 9.0.

SDS−Polyacrylamide Gel Electrophoresis and Western Blotting. SDS−polyacrylamide gel electrophoresis (SDS−PAGE) was performed using gradient gels of 8−25% acrylamide or high density gels containing 20% acrylamide on a Phast System (Pharmacia, Upsala, Sweden). Samples were diluted in 10 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA, 2.5% SDS and 0.01% bromophenol blue, and boiled for 5 min. Proteins were stained by immersing gels in 0.065% Coomassie blue R dissolved in methanol:acetic acid:glycerol:distilled water (30:10:10:50, by vol). Destaining was performed in a mixture of methanol:acetic acid:glycerol:distilled water (25:8:2:65, by vol) until a colorless background was obtained.

The Western blotting was carried out in a Milliblot-SDE system (Millipore, Billerica, MA) for 45 min at 2 mA/cm^2 of gel. The immunological detection of proteins transferred to nitrocellulose membrane (Trans Blot Medium, Bio-Rad, Hercules, CA) was performed as follows. The membrane was incubated with 5% ovalbumin in PBS for 2 h at room temperature to block nonspecific binding of proteins. After five washes with PBS, the membrane was incubated for 1 h at room temperature with antiserum to Cry1A(b) protein diluted 1:100 in PBS containing 3% ovalbumin. Membranes were washed with PBS and then incubated with HRP-conjugated goat anti-rabbit IgG (Sigma) diluted 1:500 in PBS with 3% ovalbumin. After five washes with PBS, the membranes were revealed with a solution of 3 mg of 4-chloro-1-naphthol (Merck, Darmstadt, Germany) in 1 mL of methanol, 2 mL of distilled water and 5 μL of hydrogen peroxide. The reaction was stopped by soaking the nitrocellulose membranes in distilled water.

Protein Identification by MALDI-TOF Mass Spectrometry. Bands of Cry1A(b) fraction purified by immunoadsorption obtained by SDS−PAGE were carefully cut from the Coomasie stained gel. The pieces of gel were transferred to an Eppendorf vial containing 50 μL of distilled water. The samples were analyzed at the Genomics and Proteomics Centre of the Complutense University (Madrid, Spain). Proteins selected for analysis were in-gel reduced, alkylated and digested with trypsin according to Sechi and Chait (*16*). Briefly, spots were washed twice with water, shrunk 15 min with 100% acetonitrile and dried in a Savant SpeedVac for 30 min. Then, the samples were reduced with 10 mM dithioerythritol in 25 mM ammonium bicarbonate for 30 min at 56 °C and subsequently alkylated with 55 mM iodoacetamide in 25 mM ammonium bicarbonate for 15 min in the dark. Finally, samples were digested with 12.5 ng/μL sequencing grade trypsin (Roche Molecular Biochemicals, Indianapolis, IN) in 25 mM ammonium bicarbonate buffer, pH 8.5 overnight at 37 °C. MALDI-TOF mass spectrometry analysis was performed in a 4800 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Framingham, MA). The spectrometer operated in positive reflector mode, with an accelerating voltage of 20 kV. All mass spectra were calibrated internally using peptides from the auto digestion of trypsin.

The analysis by MALDI-TOF/TOF mass spectrometry produces peptide mass fingerprints, and the peptides observed with a signal to noise greater than 20 can be collated and represented as a list of monoisotopic mass. The protein identification was accomplished by matching the observed peptide masses to the theoretical masses derived from a protein sequence database.

The band of 20 kDa, as was ambiguously identified by peptide mass fingerprints, was subjected to MS/MS sequencing analyses using the same spectrometer. From the MS spectra, suitable precursors were selected for fragmentation (MS/MS) analysis with collision-induced dissociation (CID) (atmospheric gas was used) 1 kV ion reflector mode and precursor mass Windows ± 6 Da. The plate model and default calibration were optimized for the MS/MS spectra processing.

For protein identification, the nonredundant National Centre of Biotechnology Information (NCBI) database (6189142 sequences; 2116873858 residues) was searched using MASCOT 2.1 (www.matrixscience.com) through the Global Protein Server v3.6 from Applied Biosystems. Search parameters were as follows: carbamidomethyl cystein as fixed modification and oxidized methionine as variable modification; peptide mass tolerance 50 ppm for PMF and 100 ppm for MS/MS searches; 1 missed trypsin cleavage site; MS/MS fragments tolerance 0.3 Da. The parameters for the combined search (peptide mass fingerprint and MS/MS spectra) were the same described above.

Determination of Cross Reactivity. Cross reactivity between Cry1A-(b) protein from *B. thuringiensis* and from transgenic maize was determined using an indirect competitive ELISA. Maxisorp microtritation plates (Nunc, Roskilde, Denmark) were coated with 100 μL per well of Cry1A(b) protein from *B. thuringiensis* (5 μg/mL) in 50 mM sodium carbonate buffer, pH 9.6, and incubated overnight at 4 °C. After washing the plates three times with 300 μL per well of distilled water, blocking of

residual protein binding sites was performed with 300 μL of 3% (w/v) ovalbumin in PBS at 37 °C for 2 h. After washing three times with distilled water, 50 μL of increasing concentrations of Cry1A(b) protein from *B. thuringiensis* or from transgenic maize and 50 μL of an appropriate dilution of antisera to Cry1A(b) were incubated for 1 h at 37 °C. Afterward, plates were washed five times with 0.05% Tween in PBS (PBST), and incubated with 120 μL of HRP-labeled anti-Cry1A(b) IgG in PBS for 1 h at 37 °C. After plates were washed five times with PBST, they were incubated with 100 μL per well of tetramethylbenzidine substrate (TMB, ZEU-Inmunotec, Zaragoza, Spain) for 30 min at 37 °C. Finally, the enzyme reaction was stopped by adding 50 μL per well of 2 M H_2SO_4 and the absorbance was determined at 450 nm using a microplate reader (Labsystem Multiskan, Helsinki, Finland).

The absorbance, expressed as percentage, was plotted against the concentration of each protein, and cross reactivity was calculated using the equation

$$\text{CR}\ (\%) = X_1/X_2 \times 100$$

where CR is the cross reactivity and X_1 and X_2 correspond to the concentration of Cry1A(b) protein from *B. thuringiensis* and transgenic maize, respectively, at which 50% of absorbance was obtained.

Sandwich ELISA. Maxisorp microtritation plates were coated with 100 μL per well of anti-Cry1A(b) IgG (3 μg/mL) in 50 mM sodium carbonate buffer, pH 9.6, and incubated overnight at 4 °C. After washing the plates three times with distilled water, blocking of residual protein binding sites was performed with 300 μL of 3% (w/v) ovalbumin in PBS at 37 °C for 2 h. After washing three times with distilled water, 120 μL per well of Cry1A(b) protein standards or samples in PBS was loaded in the plate in triplicate and incubated for 1 h at 37 °C. Afterward, plates were washed five times with PBST, and incubated with 120 μL of HRP-labeled anti-Cry1A(b) IgG in PBS for 1 h at 37 °C. After washing five times with PBST, plates were incubated with 100 μL per well of TMB substrate for 30 min at 37 °C. Finally, the enzyme reaction was stopped by adding 50 μL per well of 2 M H_2SO_4 and the absorbance determined at 450 nm.

Characterization of Digested Fragments by MALDI-TOF Mass Spectrometry. A volume of 1 μL of untreated or pepsin treated samples was loaded in a MALDI-TOF sample plate and allowed to dry, and then 0.5 μL of matrix, composed of sinapinic acid (10 mg/mL) in 30% acetonitrile, containing 0.3% trifluoroacetic acid was added and allowed to dry again. MALDI-TOF mass spectra (in the positive mode) were acquired on a Voyager STR mass spectrometer (Applied Biosystems, Framingham, MA) at the Complutense University (Madrid, Spain). Samples were analyzed in the linear mode using a delayed extraction (100 ns) and an accelerating voltage operating in positive ion mode of 20 kV. The spectra were calibrated externally with calmix 3 (1 pmol of insulin, thioredoxin and apomioglobin) for the low molecular mass range, and with bovine serum albumin (1 pmol) for the high molecular mass range. Average mass of proteins +1 Da was obtained, if the charge was +1 (+H), or average mass of proteins + 2 Da divided into 2, if the proteins were doubly charged (2+H).

RESULTS AND DISCUSSION

In the present work, the *in vitro* digestion of Cry1A(b) protein purified from genetically modified maize was studied. As the Cry1A(b) protein expressed in maize MON810 is the trypsin-resistant Cry1A(b) core (*17*−*19*), exclusively hydrolysis by pepsin was studied. For comparison, pepsin digestion of trypsin-activated Cry1A(b) protein purified from *B. thuringiensis* was also studied.

To perform this work, Cry1A(b) protein was isolated from fresh transgenic maize leaves, as they contain the highest concentration of this protein, about 9.35 μg/g (*12*). Purification of Cry1A(b) was performed by immunoadsorption using a column containing specific antibodies against Cry1A(b) protein insolubilized in Sepharose. Material retained in the column was eluted at acid pH, and appeared as a single peak. Fractions of this peak were pooled, and the resultant fraction was analyzed. SDS−PAGE of the resultant fraction showed several bands, with molecular weights ranging between 14 and 70 kDa (**Figure 1A**). However, Cry1A(b) protein purified from *B. thuringiensis*, used as a control, gave a single band of about 70 kDa, which is the expected mass of this protein (*18*).

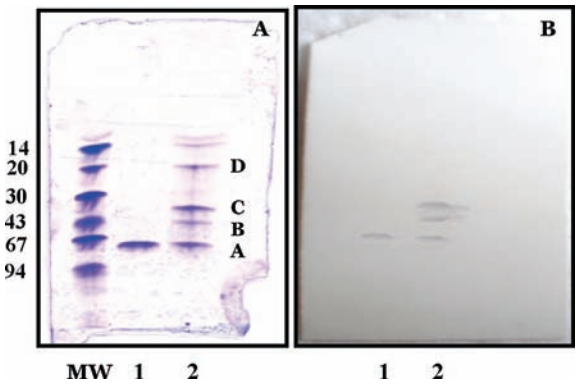

Figure 1. SDS−PAGE (**A**) and Western blotting (**B**) of Cry1A(b) protein from *B. thuringiensis* (1) and the purified fraction from transgenic maize by immunoadsorption (2) using rabbit antiserum against Cry1A(b) protein. MW: molecular weight markers (kDa).

Table 1. Results of Peptide Mass Fingerprints Analysis Obtained by MALDI-TOF Mass Spectrometry Corresponding to SDS−PAGE Bands of Cry1A(b) Fraction Purified from Transgenic Maize

	A[a]	B[a]	C[a]	D[a]
mascot score[b]	248	241	196	156
matched peptides[c]	29 (75)	28 (72)	23 (63)	7 (41)
coverage[d]/fragmented peptides[e]	50	48	38	11/3

[a] A, B, C and D, bands indicated in **Figure 1**. [b] Mascot score for the identification of proteins ($p < 0.05$). [c] Number of peptides matched to the sequence of Cry1A(b) protein, obtained from database NCBInr (ftp://ftp.ncbi.nih.gov/blast/db/FASTA/nr.gz), access number gi 36244769, and in parentheses the number of searched peptides. [d] Percentage of the sequence of amino acids that cover the peptides matched. [e] Number of fragmented peptides matching with the sequence of Cry1A(b) protein, obtained from database NCBInr, access number gi 36244769.

Afterward, proteins were transferred from the electrophoresis gel to nitrocellulose and analyzed by Western blotting using rabbit antiserum against Cry1A(b) protein. The results showed a positive reaction with the three bands of higher molecular weight obtained from Cry1A(b) fraction from transgenic maize as well as with the band of the bacterial protein (**Figure 1B**). The three bands of the maize sample that gave a positive reaction with the antiserum and the band of 20 kDa were cut from the gel and analyzed by MALDI-TOF mass spectrometry. The mass of peptides obtained from the digestion of samples was compared with the theoretical mass of tryptic peptides from proteins annotated in the NCBI database using a search engine MASCOT 2.1. Results obtained for these samples set a higher score than the Mascot score estimated for a probability < 0.05, which means that matches were not due to random event (**Table 1**). These results indicate that fragments from transgenic maize analyzed correspond to Cry1A(b) protein from *B. thuringiensis*.

The Western-blotting analysis of transgenic flours from MON810, Bt11 and Bt176 has also shown the presence of several bands (*20*, *21*). These results indicate that degradation of Cry1A-(b) by endogen proteases is occurring either in plant or during extraction after tissue breakdown. However, although hydrolysis of Cry1A(b) protein during purification could be inhibited by adding protease inhibitors, this practice is not recommended when performing proteolysis studies (*18*).

The cross reactivity between Cry1A(b) protein from *B. thuringiensis* and the purified fraction from transgenic maize was

determined by an indirect competitive ELISA using antiserum against Cry1A(b) protein (**Figure 2**). Results obtained showed that cross reactivity was about 95%, indicating that there is an immunological identity between both proteins.

Taking into account these previous results, the fraction isolated from transgenic maize was employed in this work to perform the digestibility study. To our knowledge, this is the first work carried out with Cry1A(b) protein purified from transgenic maize. Because we used this purified fraction, we could characterize treated samples by other methods besides immunochemical techniques, such as SDS−PAGE and mass spectrometry. This purified fraction was incubated with pepsin and aliquots taken at different times were analyzed. As a comparison, the degradation of Cry1A(b) protein from *B. thuringiensis* was also studied.

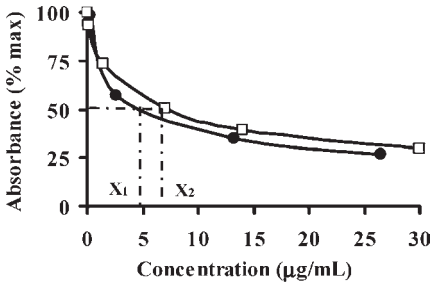

Figure 2. Cross reactivity between Cry1A(b) protein from *B. thuringiensis* (●) and Cry1A(b) fraction purified from transgenic maize by immunoadsorption (□) determined by an indirect competitive ELISA using microtiter plates coated with the bacterial protein.

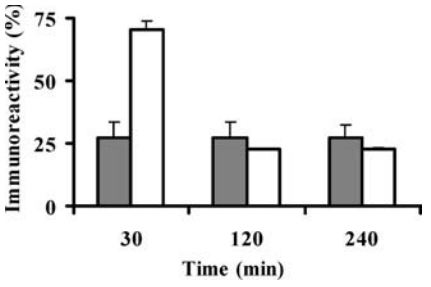

Figure 3. Remaining immunoreactive Cry1A(b) protein purified from transgenic maize (■) or from *B. thuringiensis* (□) after incubation with pepsin. Values correspond to the concentration of immunoreactive protein as determined by a sandwich ELISA, and are expressed as percentage of protein concentration in untreated samples (100%). Samples were assayed by triplicate in at least two independent experiments.

The rate of degradation of the Cry1A(b) fraction purified from transgenic maize was determined using a sandwich ELISA, previously developed (*14*, *22*). Results showed a marked decrease of immunoreactive protein to about 25% of the initial value within 30 min, this percentage remaining constant during the following 4 h of incubation (**Figure 3**). However, Cry1A(b) protein from *B. thuringiensis* was degraded by pepsin more slowly. It decreased to 70% of the initial value within 30 min, and to 23% within 2 h, this percentage remaining constant up to 4 h.

Our findings are in accordance with those previously obtained by ELISA on *in vivo* digestion of transgenic maize in pigs, calves and cows (*6*, *7*). These results indicate that the fragmented protein maintains a low but significant reactivity with the antibodies when using the ELISA technique, probably because some of them recognized fragmented yet immunoreactive parts of Cry1A(b) protein.

The ELISA technique is widely used for screening purposes, with a high throughput capacity that allows rapid and preliminary testing, and is easy to handle. However, it is not a suitable method for giving information about the appearance of potential degradation products obtained in proteolysis studies (*8*). Thus, to determine the size of Cry1A(b) protein fragments detected by ELISA, samples obtained during incubation with pepsin were also analyzed by SDS−PAGE, Western blotting and mass spectrometry.

In pepsin treated samples of Cry1A(b) from maize, the full-length protein and fragments observed in the purified fraction by SDS−PAGE were not observed at any times of treatment, and only fragments of molecular weight lower than 14 kDa and a very weak band of about 34 kDa were found when using gels of 8−25% acrylamide (**Figure 4A**). The same samples were analyzed using high density gels in order to obtain a better separation of peptides in the low molecular weight range, and bands between 6 and 8 kDa mass were observed, besides the weak band of 34 kDa (**Figure 4B**). When the sample incubated with pepsin for 30 min was analyzed by MALDI-TOF mass spectrometry covering a molecular range from 1 to 100 kDa, only peptides of lower mass than 8.5 kDa were found. **Figure 5** shows the MALDI-TOF spectra of this fraction in the range between 1.6 and 12 kDa, in which all the peaks were found. Similarly, the full-length Cry1A-(b) protein from *B. thuringiensis* was also absent in the sample incubated with pepsin at all times studied, and only a single band of approximately 34 kDa was observed by SDS−PAGE in 8−25% acrylamide gels (**Figure 4C**).

The peptides of Cry1A(b) protein from maize or from *B. thuringiensis* obtained after treatment with pepsin were transferred from the electrophoresis gel to nitrocellulose and analyzed by Western blotting using rabbit antiserum against anti-Cry1A(b).

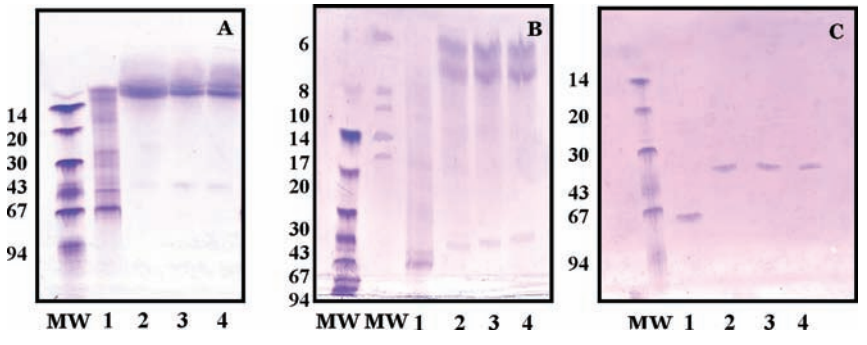

Figure 4. SDS−PAGE after digestion of Cry1A(b) protein in 8−25% (**A**, **C**) and high density (**B**) gels. Cry1A(b) fraction purified from transgenic maize (**A**, **B**) and Cry1A(b) protein from *B. thuringiensis* (**C**) after incubation with pepsin for 0 min (1), 30 min (2), 2 h (3) and 4 h (4). MW: molecular weight markers (kDa).

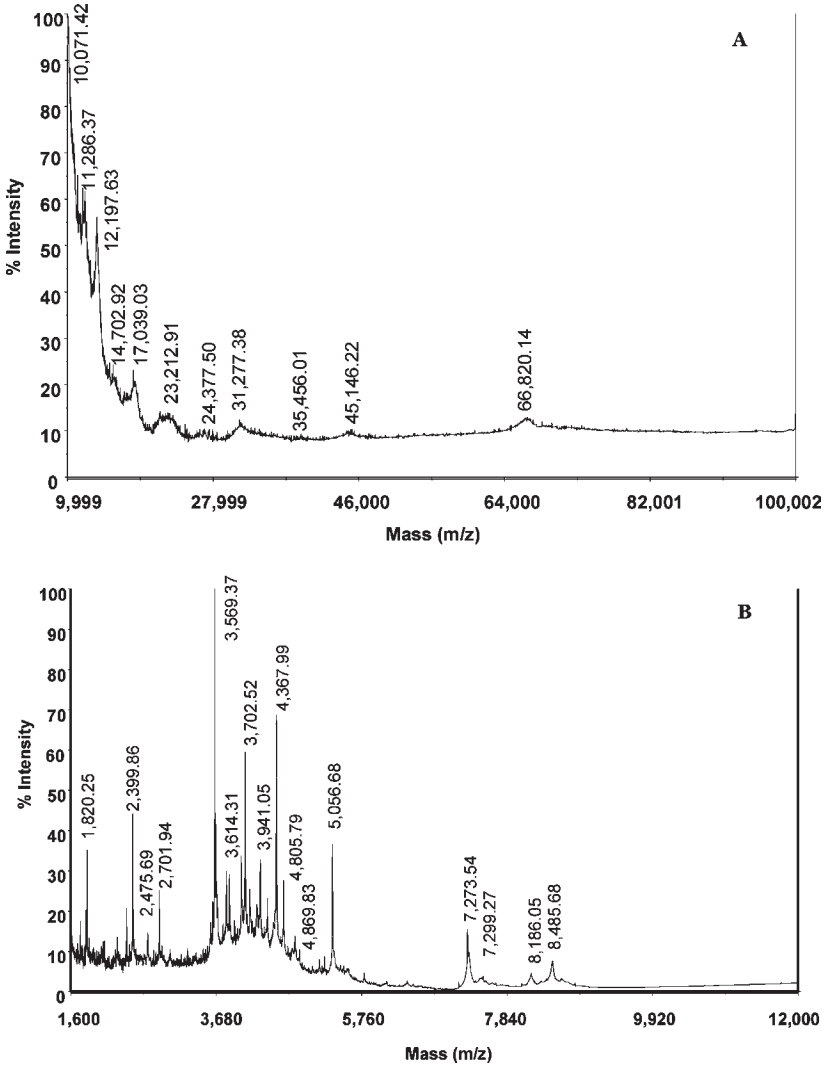

Figure 5. MALDI-TOF mass spectra of Cry1A(b) fraction purified from transgenic maize as isolated (**A**) and treated with pepsin for 30 min (**B**).

Results showed the absence of reaction in all of the samples studied (results not shown).

Our results obtained by Western blotting are in agreement with those previously reported when studying pepsin degradation of Cry1A(b) protein expressed in *E. coli* or in crude maize extracts (*11*, *13*). In these works, no reaction was observed between samples incubated with pepsin for 30 s and polyclonal (*11*) or monoclonal (*13*) antibodies against Cry 1A(b) protein when using the same technique. Results obtained in our and other works by Western blotting are in contrast with those we obtained by ELISA, and could be due to problems in the transfer of small peptides, although various transference times and voltage intensities were assayed. The lack of reaction of peptides with antibodies by Western blotting could be also due to the lower sensitivity of this technique compared to the ELISA technique.

However, in other works, the presence of Cry1A(b) protein or their fragments produced during the *in vivo* digestion of transgenic maize that react with antibodies by Western blotting has been reported (*6*−*8*). Differences in the results obtained in the proteolysis studies could be attributed to several factors such as the different maize events, the degradation conditions and the sensitivity of the antibodies used in each assay.

Our results observed by SDS−electrophoresis are in good agreement with those observed by Lutz et al. (*8*) when studying the *in vivo* degradation of Cry1A(b) protein in adult cows fed with transgenic Bt176 maize. These authors, using polyclonal or monoclonal antibodies, did not observe the presence of the full-length protein (60 kDa) in gastrointestinal contents, whereas fragments of 34 kDa and 17 kDa were visualized. As they used a gel of 4−12% of acrylamide, it is possible that the fragment of 17 kDa may correspond to peptides of lower mass, as we observed when using high density gels, which resolve peptides in the low molecular weight range, or when we determined the molecular weight of peptides by mass spectrometry.

Results obtained in this work indicate that Cry1A(b) proteins from maize or bacterial origin are rapidly degraded by pepsin. However, both samples showed a different fragmentation pattern, the maize fraction giving peptides of lower molecular weight than the bacterial protein. Differences observed between both samples could be due to that the maize protein is partially degraded by endogenous proteases, either in plant or during extraction previously to the incubation with pepsin. Therefore, the possibility that Cry1A(b) protein is already partially degraded when ingested could be considered.

Recently, much attention has been focused on foods from genetically modified plants because of the risk of allergenicity, and several works have been performed to assess it. Studies performed with sera from food allergic humans have shown that neither response to Cry1A(b) protein was observed by prick test nor detectable IgE against this protein were found in their sera (*13*, *21*).

The assessment of allergenicity can be also performed by indirect methods concerning the physicochemical properties of the foreign protein expressed, such as stability to heat or acid pH or resistance to the degradation by digestive enzymes (*23*). Obviously, the kinetics of decrease of pH and the release of digestive enzymes are difficult to reproduce, as they occur in physiological conditions. However, examination of resistance to proteolysis using standardized model systems is still considered useful information for the assessment of potential allergenicity of transgenic crops. To become allergenic, a protein must reach the intestinal tract in a form that is sufficiently intact to provoke the immune system. If the protein is rapidly digested under simulated gastric and intestinal digestive models, that prospect seems unlikely. In this sense, Cry1A(b) is a protein that shows a rapid and extensive degradation by pepsin, as has been found in this and other works, and a high sensitivity to thermal treatment (*14*, *22*, *24*), properties that are not shared by most allergenic food proteins.

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