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ARTICLE

In Vitro Digestion of Cry1Ab Proteins and Analysis of the Impact on Their Immunoreactivity

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A pepsin resistance test performed at pH 1.2 and with high pepsin to protein ratio is one of the steps of the weight-of-evidence approach used for assessment of allergenicity of new proteins. However, the use of other in vitro digestibility tests, performed in more physiologically relevant conditions and in combination with immunological assays so as to increase the value of the information gained from the studies of stability of a novel protein to digestion for the overall allergenicity assessment, has been proposed. This study then aimed to investigate the stability to digestion of Cry1Ab protoxin and toxin, insecticidal proteins expressed in genetically modified crops, using simulated gastric fluid (SGF) at different pH values and pepsin-to-substrate ratios, in the presence or absence of physiological surfactant phosphatidylcholine (PC). Electrophoresis and immunoblot patterns and residual immunoreactivity of digesta were analyzed. Although Cry1Ab protoxin is extensively degraded at pH 1.2 with high pepsin-to-protein ratio, it is only slightly degraded at pH 2.0 and conserved its immunoreactivity. Furthermore, Cry1Ab proteins were demonstrated to be stable in a more physiologically relevant in vitro digestibility test (pH 2.5, pepsin-to-substrate ratio 1:20 (w/w) with PC). Factors such as pH, SGF composition, and pepsin-to-substrate ratio then greatly influence the digestion of Cry1Ab proteins, confirming that new and more physiologically relevant in vitro digestibility tests should be also considered to study the relationship between the resistance of a protein to digestion and its allergenicity.

KEYWORDS: Cry1Ab; *Bacillus thuringiensis*; allergenicity assessment; digestion; immunoreactivity

1. INTRODUCTION

Food allergies, mainly IgE-mediated reactions, are increasing worldwide. The prevalence of allergy to individual foods varies geographically due to variation in dietary practices, and many foods may be incriminated (*1*, *2*). The introduction of novel foods and foods derived from biotechnology has therefore raised the question of their potential allergenicity. Before introduction on the market, genetically modified (GM) crops are then subjected to extensive assessment of their potential effects on human and animal health, including toxicity and allergenicity. As no single test or property allows distinguishing allergenic from nonallergenic proteins, food allergy risk assessment of newly expressed proteins in a GM crop is based on a weight-of-evidence approach (*3*−*5*) including, among a variety of tests, the study of stability during the digestion process (i.e., pepsin resistance test).

Resistance to digestion has been reported to be a property shared by some of the dietary proteins known to sensitize atopic patients by the gastrointestinal route (*6*). It is thus suggested that a protein at least partially stable to the proteolytic and acidic conditions of the digestive tract has an increased probability of reaching the intestinal mucosa in a form that is sufficiently immunological active to sensitize the mucosal immune system. In the past decade several in vitro models have been developed to evaluate the stability of proteins to digestion (*7*). A standardized digestibility model was proposed by a multilaboratory consortium (*8*) using pepsinolysis protocols and simulated gastric fluids (SGF) that are also established for preclinical testing of pharmaceuticals, as described by the U.S. Pharmacopeia (*9*). The ability of this test initially described as allowing nonallergens and allergens to be distinguished (*10*) has been reassessed for the consideration of more test proteins and the study of the effect of pH, enzyme-to-substrate ratio, and inclusion of physiologically relevant surfactants (*11*−*16*).

Cry1A protoxins are 130 kDa proteins naturally produced by *Bacillus thuringiensis*, a soil Gram-positive bacterium. The N-terminal moiety of protoxin contains the toxic part of the molecule, called δ-endotoxin or toxin. It corresponds to a 65 kDa product of cleavage, which is produced in insect larvae gut and

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kills them (*17*). Within Cry1A proteins, Cry1Ab is expressed as (part of) protoxin in maize MON 810 or toxin in maize Bt11 or Bt176 (http://www.agbios.com, Australia New Zealand Food Authority Draft risk analysis report, application A386, October 2000), although the exact sequences inserted are not disclosed. Several Cry proteins and particularly Cry1Ab have been extensively reviewed by EFSA in terms of food and feed safety and allergenicity within the framework of assessment of applications for the approval of insect-resistant (IR) crops (*18*, *19*). The rapid and complete degradation of Cry1Ab that was observed when the protein was subjected to the pepsin resistance test was part of the information used to conclude that the allergenicity of Cry1Ab expressed in IR crops is unlikely. It is not our aim to question this conclusion based on a weight-of-evidence approach but rather to discuss the procedures used for in vitro digestibility testing and their impact on both the degradation of the protein and the immunoreactivity of the resulting fragments.

We then compared in vitro digestibility of Cry1Ab proteins using a "high-protease assay" performed at low pH values and high pepsin-to-protein ratio as previously reported by several authors and validated within a multilaboratory evaluation (*8*−*12*) and a more physiologically relevant test, also recently validated within a multilaboratory evaluation (*7*, *14*, *15*). Although pepsin secretion and digestion efficiency may be highly variable between individuals, suggesting that in vitro models would never mimic in vivo digestion, this latter test is considered to be more physiologically relevant due to enzyme-to-substrate ratio and pH value applied and consideration of the multiphase nature of stomacal chyme due in part to lipid emulsion. In fact, estimation of pepsin secretion and protein intake indicates that the amount of protein would normally exceed that of pepsin, then suggesting that "high-protease assay" does not reproduce ratios occurring in vivo (*14*). Moreover, the pH in the stomach after meal ingestion can drop to pH 2−2.5, but it can also increase to values around 3. Conversely, when the stomach is empty, the pH can be as low as 1.5 (*7*). Altogether, these data suggest that in vitro digestion at pH 1.2 is far from physiological conditions. Several studies have also demonstrated the importance of developing "multiphase" digestion models, reproducing interactions of proteins with physiologically relevant levels of phospholipids such as phosphatidylcholine (PC), which can alter the susceptibility to pepsin (*14*, *15*, *20*). Those constituents are abundant in some foods such as milk, but are also actively secreted by gastric mucosa: PC in the gastric mucus constitutes a hydrophobic protective layer (*21*). To analyze in vitro pepsinolysis of Cry1Ab protoxin and toxin, we then compared the electrophoretic patterns obtained after digestion of well-characterized bacteria-derived proteins using different pH values and pepsin-to-protein ratios and in the presence or absence of physiological surfactant (PC). To assess their residual immunoreactivities, digesta were further analyzed with monoclonal or polyclonal antibodies raised against the entire protoxin or its N-terminal portion, that is, toxin. IgE and IgG binding capacities of the final digesta were finally studied using sera from mice experimentally sensitized to Cry1Ab protoxin.

2. MATERIALS AND METHODS

2.1. Reagents. Unless otherwise stated, all reagents were of analytical grade from Sigma (St. Louis, MO).

2.2. Protein Production and Purification. *Recombinant Cry1Ab Protoxin Production*. Recombinant Cry1Ab protoxin was produced in *Escherichia coli* JM103 carrying the expression vector pKK223-3:cry1Ab kindly provided by D. R. Zeigler, BGSC (Ohio State University). Recombinant *E. coli* were grown in 500 mL of Luria broth medium under agitation (48 h at 37 °C). After centrifugation, the bacterial pellet was resuspended in 50 mL of TESL buffer (50 mM Tris-HCl pH 8, 50 mM EDTA, 15% sucrose, 2 mg/mL lysozyme) with protease inhibitors (50 μg/mL bacitracin, 300 μg/mL benzamidin, 20 μg/mL leupeptin, 20 μg/mL chymostatin, 2.5 μg/mL pepstatin A, 60 μg/mL 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride). After incubation overnight at 4 °C under agitation, lysate was sonicated and centrifuged. The pellet was rinsed twice in 0.5 M NaCl, 2% Triton X-100, and then with distilled water. Protein crystals were dissolved in bicarbonate/dithiothreitol (DTT) buffer (50 mM Na_2CO_3/HCl, pH 9.5, 10 mM DTT). After centrifugation, the supernatant containing the recombinant Cry1Ab protoxin was dialyzed overnight at 4 °C against 50 mM carbonate buffer, pH 9 (MW cutoff 12000−14000 Da, Spectra/Por molecular porous membrane tubing; Spectrum Laboratories, Los Angeles, CA), thus allowing protease inhibitor removal (MW < 1000). The further purification of protoxin was performed by size exclusion chromatography coupled to an Akta purifier system using Sephacryl S100 HR column (both from Amersham Pharmacia Biotech) and 20 mM piperazin, 0.5 M NaCl buffer. The proteins were detected by UV absorbance at 280 nm, providing two purified fractions (a and b, **Figure 1A**). Corresponding fractions were further dialyzed against carbonate buffer (20 mM, pH 9.6) using a Slide-a-Lyzer dialysis cassette (3500MWCO, Pierce), dispatched, and kept frozen until use (−80 °C). Fractions were analyzed by electrophoresis and Western blot experiments using monoclonal antibody mAb 120 (**Figure 1B** and section 2.6), the same as the dialyzed supernatant collected before size exclusion chromatography (S, **Figure 1B**). Separated electrophoretic bands from fraction a presenting molecular masses close to 250 and 130 kDa and between 98 and 130 kDa were further analyzed by in gel digestion with porcine trypsin (Promega), as previously described (*22*). Generated fragments were characterized by mass spectrometry (MALDI-TOF, Voyager DE-RP instrument, Applied Biosystems), and databases were searched online with the Profound and Protein Prospector tools available at www.expasy.ch. This analysis confirmed that the protein band close to 130 kDa corresponded to Cry1Ab protoxin (accession no. 117533, 1155 amino acids, MW 130625 Da), with 36−40% of protein sequence coverage from amino acids 1 to 1110, which is close to previous characterization of Cry proteins (*23*). Protein of 250 kDa then corresponds to Cry1Ab dimer, and protein bands observed below 130 kDa could not be differentiated from whole protoxin; that is, no C-terminal or N-terminal sequences were missing. A specific immunoblot experiment using antitoxin polyclonal antibodies also demonstrated that the 60 kDa band corresponds to Cry1Ab toxin, likely produced in *E. coli* after bacterial cleavage of protoxin (data not shown). All in vitro pepsin digestion experiments were further performed on fraction a, corresponding to the purified Cry1Ab protoxin.

Cry1Ab Toxin Production from B. thuringiensis. The *B. thuringiensis* 407 strain harboring the pHT315Ωcry1Ab plasmid (Vincent Sanchis, unpublished results) overproducing the Cry1Ab protoxin was grown in 100 mL of HCT medium (*24*) at 37 °C, 175 rpm, until sporulation was completed. The culture pellet was washed twice with water and loaded on a 31−80% two-phase discontinuous sucrose gradient and spun down at 37000*g* for 90 min at room temperature. Parasporal bodies were recovered at the 31−80% sucrose interface and extracted during 60 min at 37 °C with 0.1 N NaOH, 10 mM EDTA, and 1 mM PMSF. The solubilized Cry1Ab protoxin was then precipitated by decreasing the pH to 6.0 using small volumes of 1.5 M Bis-Tris, 20 mM EDTA, and 1 mM PMSF, pH 2.0. Protoxin was then cleaved using trypsin, and the fragment corresponding to the 65 kDa δ-endotoxin Cry1Ab was further purified and analyzed by electrophoresis (**Figure 1C**) and in-gel digestion with porcine trypsin, as described previously. This analysis confirmed that the purified protein corresponded to the N-terminal part of Cry1Ab protoxin (accession no. 117533), the matched peptides covering 43.8% of the protein sequence from amino acids 1 to 700.

β-Casein Purification. β-Casein (molecular mass 26000 Da) was purified from cow's milk as previously described (*25*, *26*) and electrophoresis analysis performed as described under section 2.4 (**Figure 1D**).

2.3. Cry1Ab-Specific Monoclonal and Polyclonal Antibodies. Anti-Cry1Ab protoxin monoclonal antibodies (mAbs) were produced by conventional techniques (*27*, *28*). Briefly, 30 μg of Cry1Ab protoxin emulsified in Freund's complete adjuvant was injected into the foot pad of four female Biozzi high-responder mice on days 0 and 21. The mice producing the highest titers of specific antibodies received two intravenous booster injections, and their spleen cells were fused with NS1 myeloma cells 3 days later. The presence of anti-Cry1Ab antibodies in supernatants of hybridoma cells was tested 10 days later by checking their capacity to

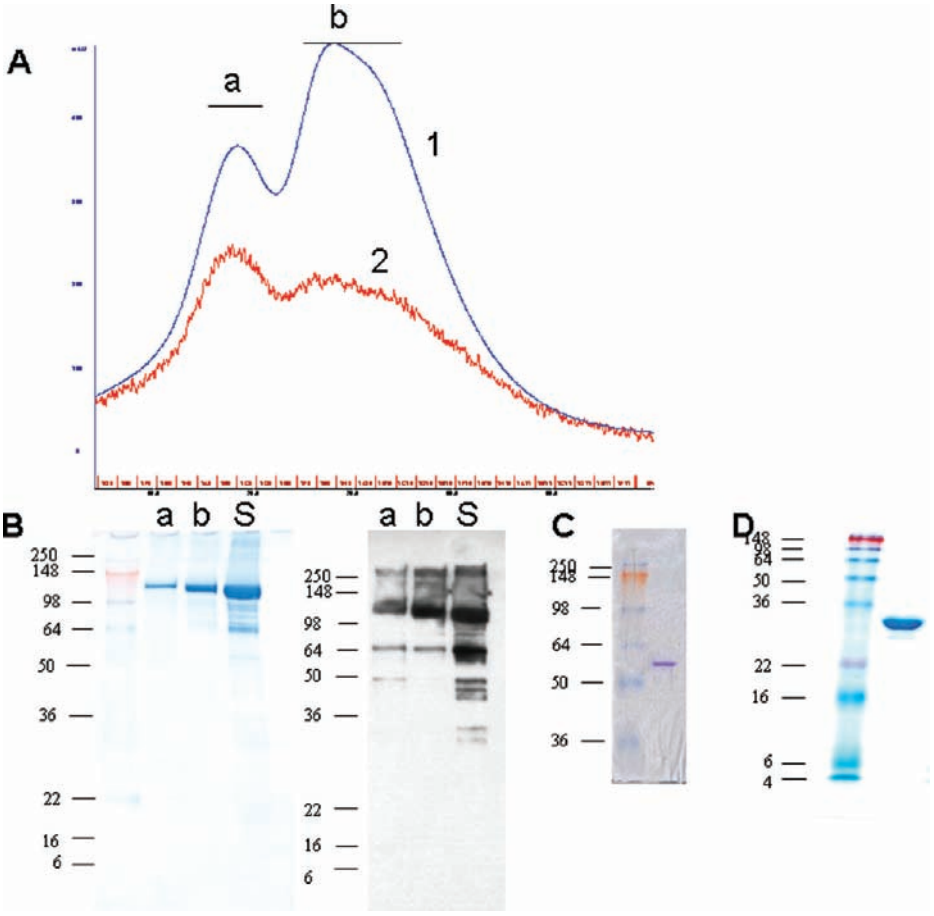

Figure 1. Purified proteins used for digestibility tests. (**A**) Cry1Ab protoxin was produced in *E. coli* JM103 carrying the expression vector pKK223-3:cry1Ab. The final bacterial extract containing the recombinant Cry1Ab protoxin was purified by size exclusion chromatography. The proteins were detected by UV absorbance at 280 nm (line 1) and 220 nm (line 2), providing two purified fractions (a and b). (**B**) Corresponding fractions were analyzed by electrophoresis (left panel) and Western blotting using mAb 120 (right panel) on Novex Tris-Gly 10−20% gel using provided electrophoresis, transfer systems, and buffers and following manufacturer's recommendations (Invitrogen). Lanes: a and b, purified fractions a and b, respectively; S, bacterial supernatant before size exclusion chromatography. MW, SeeBlue Plus2 Prestained standard marker (Invitrogen). (**C**) Electrophoresis of Cry1Ab toxin purified from *B. thuringiensis*. (**D**) Electrophoresis of β-casein purified from cow's milk.

bind Cry1Ab biotinylated using activated *N*-hydroxysuccinimidine ester of biotin as previously reported (*29*). The relative affinity of the monoclonal antibodies was estimated by competitive enzyme immunoassay (EIA) in microtiter plates coated with goat anti-mouse antibodies (Jackson Immuno Research Laboratories., West Grove, PA) (*29*), and the mAbs presenting the highest affinity (n = 25) were expanded as ascitic fluid in nu/nu mice and purified using caprylic acid precipitation (*30*). Binding complementarity, that is, the simultaneous binding of two different mAbs to different regions of a single Cry1Ab molecule, was tested in sandwich immunoassay. Those assays used microtiter plates coated with one mAb as capture antibody and all other biotinylated mAbs as tracer antibodies in the presence of various concentrations of Cry1Ab. MAbs applied as tracers that were unable to bind the Cry1Ab protein immobilized by the capture mAb were classified in the same group of binding compatibility as the immobilized mAb. On the contrary, mAbs binding the Cry1Ab protein in the presence of the first mAb defined a new group of binding compatibility. Five complementarity groups were characterized, one of which is specific for the C-terminal part of the protoxin.

Polyclonal antibodies to Cry1Ab toxin were raised in Bouscat rabbits (WISS, St Savine, France). Rabbits were immunized subcutaneously five times at monthly intervals with 200 μg of toxin emulsified in complete Freund's adjuvant. Antisera, containing polyclonal antitoxin antibodies (pAbs), were obtained from bleeds collected 1 week after the three last immunizations.

2.4. Cry1Ab Protoxin and β-Casein Digestion Using the Pepsin Resistance Test as a "Standard" in Vitro Gastric Digestion Model. The model used as a "standard" gastric digestibility test, or "high-protease level assay" (*15*), performed in this study has been previously described (*8*). Briefly, the test protein was dissolved in standard simulated gastric fluid (sSGF; 0.084 N HCl, 35 mM NaCl; pH 1.2 or 2.0). After incubation at 37 °C for 15 min, a solution of 0.32% (w/v) porcine pepsin (EC 3.4.23.1, activity = 3440 U/mg of protein calculated using hemoglobin as substrate) in sSGF was added. The final gastric digestion solution thus corresponded to an enzyme-to-protein ratio of 3:1 (w/w), giving 10 U of pepsin/μg of Cry1Ab. The digestion was performed at 37 °C under agitation (170 rpm), and 200 μL aliquots were taken at 0.5, 1, 2, 5, 10, 20, and 60 min of digestion for further analysis. The digestion was immediately stopped in corresponding aliquots by the addition of 70 μL of 0.2 M ammonium bicarbonate. Positive control of digestion were performed under the same experimental conditions, using β-casein as a control protein (*7*). Analysis of digesta was performed by electrophoresis: 15 μL of samples was added to 5 μL of 4× Laemmli SDS-PAGE loading buffer and heated at 90 °C for 10 min. Samples were then loaded onto 15% SDS-PAGE using Mini-Protean II cell system from Bio-Rad (Hercules, CA), following the manufacturer's instructions. All gels were stained using GelCode Blue Reagent (Thermo Scientific, Rockford, IL).

2.5. Cry1Ab Proteins and β-Casein Digestion Using a More Physiologically Relevant in Vitro Gastric Digestion Model. The physiologically relevant gastric digestion model used in this study has been previously described and validated (*14*, *15*). Briefly, digestions were performed in the absence or presence of phospholipid vesicles (L-α-phosphatidylcholine, PC). For the former, Cry1Ab proteins (protoxin and toxin) were dissolved in physiological simulated gastric fluid (pSGF, 0.15 M NaCl, pH 2.5, adjusted with 1 M HCl). In the latter case, Cry1Ab

proteins were dissolved in pSGF and mixed with PC vesicles (1:1.2, v/v) (*15*). The final concentration of PC was 6.3 mM. After incubation at 37 °C for 15 min, a solution of 0.32% (w/v) porcine pepsin (EC 3.4.23.1, activity = 3440 U/mg of protein) in pSGF was added. The final gastric digestion mix corresponded to a pepsin-to-protein ratio of 1:20 (w/w), giving 172 U of pepsin/mg of Cry1Ab. Two other pespin-to-protein ratios were also tested: 1:1 (w/w), giving 3440 U of pepsin/mg of Cry1Ab, and 20:1 (w/w), giving 68800 U of pepsin/mg of Cry1Ab. The digestion was performed at 37 °C under agitation (170 rpm), and 100 μL aliquots were taken at 0.5, 1, 2, 5, 10, 20, and 60 min of digestion for further analysis. The digestion was immediately stopped by the addition of 20 μL of 0.5 M ammonium bicarbonate. Positive control of digestion using β-casein was also performed for all three ratios. Analysis of digestion products was performed by electrophoresis: 5 μL of sample digesta was mixed with 5 μL of Tris−glycine SDS sample buffer (2×) and 1 μL of NuPAGE reducing agent (1×) and heated at 85 °C for 2 min. Electrophoresis was performed by loading 10 μL of sample in a 4−20% gradient Novex Tris−glycine gel (all from Invitrogen) at 125 V for 100 min.

2.6. Immunoblotting of Toxin and Protoxin Digesta. Electrophoresis of 5 μL of digesta samples was performed as described under section 2.5. Proteins were transferred onto a PVDF membrane using X-cell blot module according to the manufacturer's recommendations (Invitrogen). Membrane was then incubated either with anti-protoxin mouse monoclonal antibody (mAb 120, final concentration = 1 μg/mL) or with anti-toxin polyclonal antibodies (1:50000 dilution of rabbit antisera) for 18 h at 4 °C. After washing, corresponding secondary antibody solutions were added (Amersham ECL anti-mouse or anti-rabbit IgG, horseradish peroxidase-linked antibody, 1:5000 dilution). For signal detection, membranes were incubated with ECL plus Western Blotting detection reagent (Amersham). Signals were visualized by exposing a detection film to the blots further developed with Kodak X-film developer.

2.7. Competitive Immunoassays of Cry1Ab Protoxin Digesta. To assess immunoreactivity of protoxin digesta, competitive immunoassays were performed between protoxin and its digesta for binding to monoclonal antibodies raised against the protoxin. Enzyme immunoassays were performed in 96-well microtiter plates (Immunoplate Maxisorb, Nunc, Roskilde, Denmark) using specialized Titertek microtitration equipment from Labsystems (Helsinki, Finland). Different monoclonal antibodies (5 μg/mL, in 50 mM phosphate buffer, pH 7.4), recognizing different regions on the protoxin (i.e., belonging to complementarity groups 1−5), were passively adsorbed on microtiter plates. Fifty microliters of biotinylated protoxin (biotin EZ-link from Pierce, 20 mol of biotin/mol of Cry1Ab) was then incubated at the same time as 50 μL of concentrations from 0.3 to 5 μg/mL of competitors, that is, nondigested or digested protoxin, for 18 h at 4 °C. All samples were diluted in EIA buffer (0.1 M phosphate buffer, 0.1% bovine serum albumin, 0.15 M NaCl, 0.01% sodium azide). Plates were then extensively washed, and acetylcholinesterase (AChE)-labeled streptavidin was added for 3 h. Solid-phase bound AChE activity was determined by addition of 200 μL/well of Ellman's medium and 414 nm absorbance measurement (*31*). Data are expressed as B/B_0 values, where B_0 corresponds to the absorbance obtained in the absence of competitor and B to that obtained in the presence of competitor.

2.8. IgE-Binding Capacities of Cry1Ab Protoxin Digesta. Five-week-old female Balb/cJ mice (Centre d'Elevage René Janvier, France) were housed under normal husbandry conditions and were acclimated for 2−3 weeks before immunizations. All experiments were performed according to the European Community rules of animal care and with permission 91-122 of the French Veterinary Services. To induce a high anti-protoxin IgE response, mice were experimentally sensitized on days 1 and 14 by intraperitoneal administration of 5 μg of Cry1Ab protoxin emulsified in incomplete Freund's adjuvant (IFA, Difco, Detroit, MI), according to the method of Adel-Patient et al. (*32*). Serum samples were collected on day 18 and protoxin-specific IgE and IgG1 titers assessed on protoxin-coated plates (10 μg/mL in 50 mM phosphate buffer, pH 7.4) using AChE-labeled rat anti-mouse IgE (clone LOME3, Serotech, U.K.) or AChE-labeled goat anti-mouse IgG1 (Southern Biotechnology Associated, Birmingham, AL), as already described (*33*). IgE and IgG1 binding capacities of protoxin digesta were assessed following the same procedure, using plates coated with the digesta (10 μg/mL in 50 mM phosphate buffer, pH 7.4) and convenient dilution of pooled sera from protoxin-sensitized mice. Serum incubation was performed for 18 h at 4 °C, and, after washing, enzymatic tracers were added onto the plates for 3 h at room temperature. Each point was performed in triplicate, and nonspecific binding obtained with sera from naïve mice on the different digesta time points coated wells were subtracted from that obtained with immune sera.

3. RESULTS

3.1. Cry1Ab Protoxin Digestion Using the Pepsin Resistance Test as a "Standard" in Vitro Gastric Digestion Model. *3.1.1. Digestibility Assay of Cry1Ab Protoxin.* "Standard" in vitro gastric digestion of Cry1Ab protoxin using simulated gastric fluid (sSGF) at pH 1.2 and 2.0 and a pepsin-to-protein ratio of 3:1 was performed, and digesta obtained at different time points were analyzed by electrophoresis. Analysis of samples taken at times ranging from 0.5 to 60 min demonstrated that Cry1Ab is rapidly degraded at pH 1.2, showing no fragment even at the first time point (t = 0.5 min) (**Figure 2A**). Using sSGF at pH 2.0, Cry1Ab was only slightly degraded and still visible as intact protein after 60 min (**Figure 2A**). The efficiency of this in vitro digestion model was confirmed using bovine β-casein as a highly pepsin-sensitive protein. As expected, β-casein was extensively degraded as quickly as 1 min after pepsin addition at pH 1.2 (**Figure 2B**, left panel) (*7*). Digestion was slower at pH 2.0, but no intact β-casein was detected after 10 min of digestion (**Figure 2B**, right panel).

Altogether, these results indicate that Cry1Ab protoxin is highly degraded by pepsin at pH 1.2 but is only partially degraded when the pH is increased to 2.0, whereas milk β-casein, used as a control, was extensively degraded at both pH values.

3.1.2. Cry1Ab Protoxin Digesta Immunoreactivity. Samples obtained at times ranging from 0.5 to 60 min in the standard in vitro model were blotted using anti-Cry1Ab protoxin monoclonal antibody. **Figure 3A** shows the Western blot digestion profile when using sSGF at pH 1.2. Blotting of highly purified protoxin before digestion showed additional slight bands also identified as Cry1Ab proteins (see Materials and Methods). In all cases, we observed a significant degradation of Cry1Ab protoxin after the addition of pepsin, as evidenced by the appearance of low molecular weight (LMW) bands from t = 0.5 to t = 5 min, whereas full-length protoxin progressively disappeared. It is worth noting that the 130 kDa full-length protoxin was still detected after 60 min of digestion using Western blotting, although it was not detectable in gel electrophoresis after staining (**Figure 2A**), which thus likely corresponds to very low amounts of intact protein. After digestion using sSGF at pH 2.0, samples taken at times ranging from 0.5 to 60 min evidenced a slight degradation of the 130 kDa fragment from t = 0.5 min (data not shown). These results confirm those obtained by electrophoresis analysis showing that Cry1Ab protoxin is extensively degraded after pepsin digestion in sSGF at pH 1.2, but is only slightly degraded when the pH value is increased to 2.0.

3.1.3. Cry1Ab Protoxin Digesta Immunoreactivity Using Anti-Cry1Ab Protoxin IgE and IgG1 Raised in Mice. The IgE and IgG1 binding capacities of the protoxin digesta were assessed using sera from mice experimentally sensitized with protoxin and compared to that of purified protoxin maintained in basic conditions. As demonstrated in **Figure 3B**, the IgE and, to a lesser extent, IgG1 binding capacities of Cry1Ab protoxin subjected to strong acid conditions (i.e., pH 1.2 or 2.0) and then neutralized are highly decreased even before digestion. Analysis of digesta obtained at pH 1.2 demonstrated a total loss of IgE and IgG1 binding capacity as quickly as 2 min after pepsin digestion. Conversely, the IgE and IgG1 binding capacities of Cry1Ab protoxin digested at pH 2.0 for at least 60 min are comparable to those observed before pepsin addition.

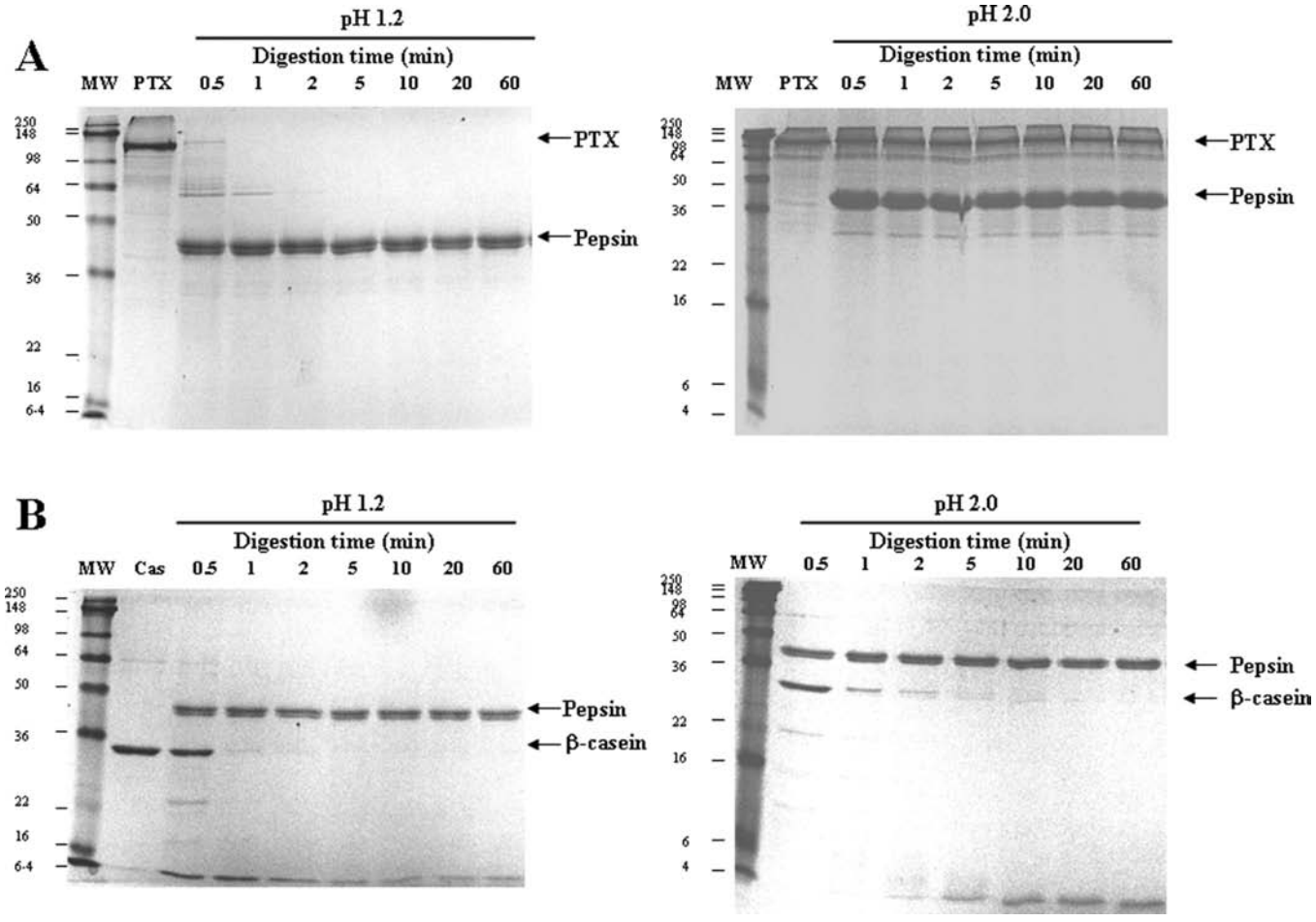

Figure 2. Cry1Ab protoxin resistance to pepsin digestion at pH 1.2 and 2.0. Protoxin (PTX; **A**) or β-casein (**B**) was submitted to a pepsin resistance test at pH 1.2 (left panel) or pH 2.0 (right panel) and pepsin-to-protein ratio 3:1 (w/w). Aliquots were taken at 0.5, 1, 2, 5, 10, 20, and 60 min after the start of pepsinolysis and were loaded on 15% gel (protoxin, 1.9 μg/well; β-casein, 375 ng/well). Protoxin, pepsin, and β-casein proteins are indicated. MW, SeeBlue Plus2 Prestained standard marker (Invitrogen).

3.2. Cry1Ab Proteins Digestion Using an in Vitro Digestibility Test Performed in More Physiological Conditions. *3.2.1. Digestibility Assay of Cry1Ab Protoxin.* In vitro gastric digestion of Cry1Ab protoxin was next performed using pSGF at pH 2.5 and a pepsin-to-protein ratio of 1:20 (w/w), in the absence or presence of PC. Two others ratios (1:1 and 20:1) were also tested, although they are less physiologically relevant. At a pepsin-to-protein ratio of 1:20, analysis of samples taken at times ranging from 0.5 to 60 min did not evidence any change in electrophoresis profiles, even after 60 min of pepsin digestion, regardless of the absence or presence of PC (**Figure 4A**). For pepsin-to-protein ratios of 1:1 and 20:1, no change in electrophoresis profile digesta could be evidenced, except after 60 min of pepsin digestion, when degradation fragments were evidenced. At this time point, an increasing quantity of pepsin increased the intensity of degradation (**Figure 4A**). However, we observed that PC protected the protoxin from the digestion at these high pepsin-to-protein ratios. In fact, intact protoxin was no more detectable after 60 min of digestion at a pepsin-to-protein ratio 20:1 in the absence of PC, whereas it was still visible in the presence of PC (**Figure 4A**). These results suggest that Cry1Ab protoxin is resistant to pepsin digestion using pSGF at pH 2.5, but increasing digestion time and pepsin concentration and using PC affected digestion profile.

The efficiency of the in vitro digestion model was confirmed using β-casein, which was rapidly degraded a few minutes after pepsin addition for pepsin-to-protein ratios of 1:20 (**Figure 4B**) (*15*), 1:1, and 20:1 (data not shown).

3.2.2. Cry1Ab Protoxin Digesta Immunoreactivity Using Monoclonal Antibodies. Samples collected after 60 min of in vitro pepsin digestion at pH 2.5 at the different pepsin-to-protein ratios were blotted using the anti-Cry1Ab protoxin mAb120 and compared to that of purified untreated Cry1Ab protoxin (**Figure 5A**). We then confirmed the increase of degradation products when the pepsin concentrations increased, and we evidenced the protective effect of PC on the 60 min digesta at all ratios considered.

We then assessed the immunoreactivity of the digesta by performing competitive immunoassays between biotin-labeled protoxin and purified protoxin (not submitted to acidic pH) or protoxin digesta obtained after 60 min of pepsin digestion without PC. As compared to the purified protein, a moderate loss of immunoreactivity of the digesta was observed when using mAbs specific for either the C-terminal (i.e., mAb101) or the N-terminal (i.e., mAb39) part of the protoxin (**Figure 5B**). Surprisingly, immunoreactivity of 60 min digesta was slightly higher for pepsin-to-protein ratios of 1:1 and 20:1 when compared to a ratio of 1:20, although less degradation was observed for the latter ratio. The same results were obtained for all monoclonal antibodies tested, recognizing different parts of the protein (n = 5, data not shown). Nevertheless, this loss of immunoreactivity is mostly due to the acidic pH (i.e., pH 2.5) of the SGF, because we observed a nearly comparable loss of the immunoreactivity of the protoxin in SGF, pH 2.5, without pepsin (data not shown and see below), as previously observed for pH 1.2 and 2 (**Figure 3B**).

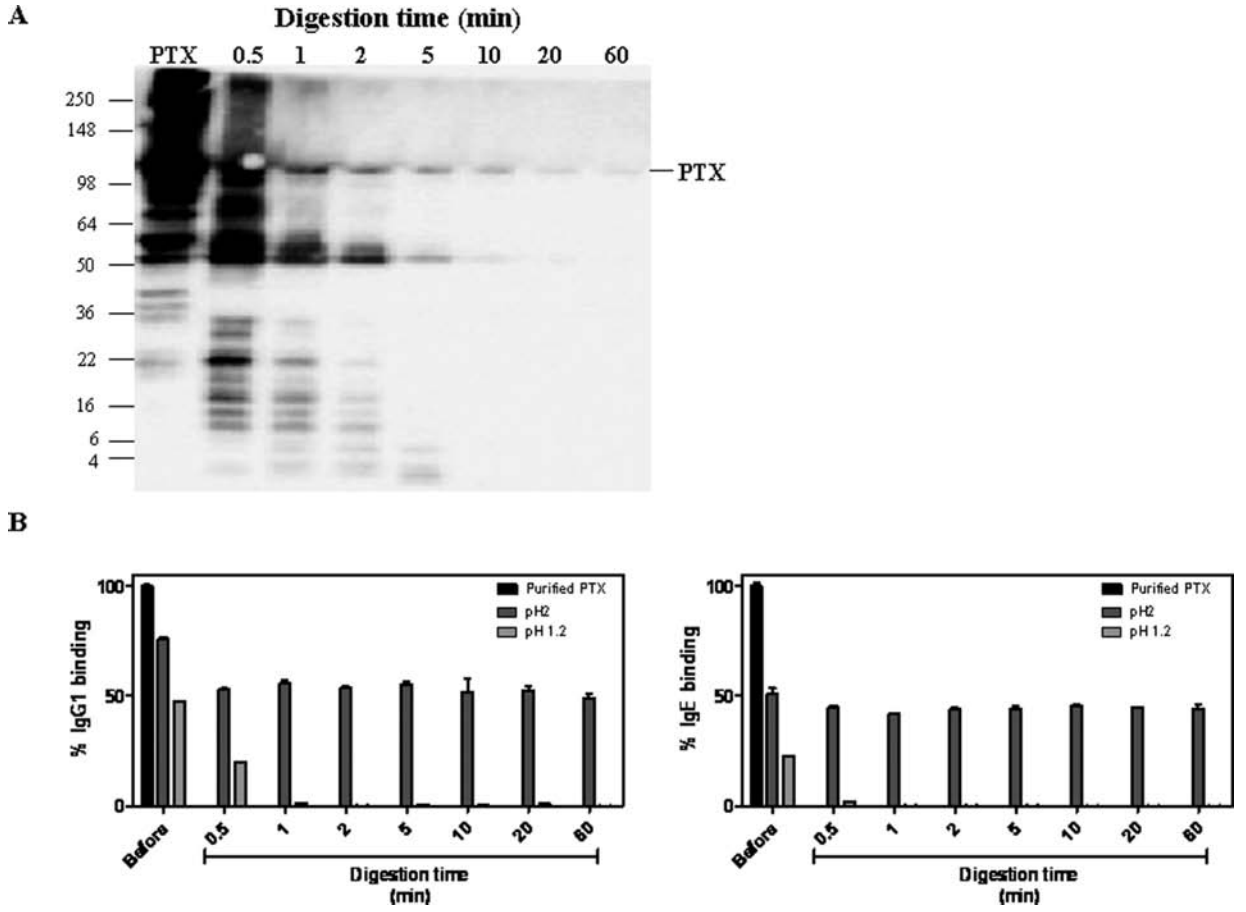

Figure 3. Immunoreactivity and IgE and IgG1 binding capacity of Cry1Ab protoxin digesta obtained after pepsin resistance test. (**A**) Samples taken at 0.5, 1, 2, 5, 10, 20, and 60 min after the start of pepsin resistance test at pH 1.2 were blotted with the anti-Cry1Ab protoxin monoclonal antibody 120. Purified Cry1Ab protoxin (PTX) is indicated. (**B**) Digesta were passively immobilized onto microtiter plates. IgG1 (left panel) and IgE (right panel) binding capacities were assayed using pooled sera from mice experimentally sensitized with Cry1Ab protoxin. Sera from nave mice were analyzed on the same digesta, giving nonspecific binding values that were subtracted from that obtained with immune sera. Sera were diluted 1/50 and $1/10^6$ for IgE and IgG1 detection, respectively, and assayed in triplicates for each digestion time. Reference signal (100%) was obtained using immobilized purified protoxin not submitted to acidic conditions (black bars). "Before" indicates protoxin diluted in SGF at pH 1.2 or 2.0, without pepsin.

3.2.3. Cry1Ab Protoxin Digesta Immunoreactivity Using Anti-Cry1Ab Protoxin IgE and IgG1 Raised in Mice. The IgE and IgG1 binding capacities of the protoxin digesta were assessed using sera from mice experimentally sensitized with protoxin and compared to those of purified protoxin maintained in basic conditions (pH 9). As demonstrated in **Figure 5C**, the IgE and IgG1 binding capacityies of Cry1Ab protoxin were reduced after dilution in SGF at pH 2.5. An additional slight decrease was observed when different digestion time points were considered, as demonstrated in **Figure 5C** using a pepsin-to-protein ratio of 1:1 without PC. As previously observed, IgE and IgG1 binding capacities were higher for pepsin-to-protein ratios of 1:1 and 20:1 as compared to a ratio of 1:20 (data not shown).

3.3. In Vitro Gastric Digestion of Cry1Ab Toxin. *3.3.1. Digestibility Assay of Cry1Ab Toxin*. In vitro digestibility test of Cry1Ab toxin by pepsin was assessed in pSGF (pH 2.5) as described for Cry1Ab protoxin. Samples taken after 60 min of digestion were first analyzed by SDS-PAGE (**Figure 6A**). For pepsin-to-protein ratios of 1:20 and 1:1, we observed a degradation product of approximately 25 kDa (**Figure 6A**), but the intact toxin persisted even after 60 min of pepsin digestion, as evidenced by the presence of the 65 kDa band. For a pepsin-to-protein ratio of 20:1, degradation of toxin was more intense and three degradation products of approximately 23, 25, and 30 kDa were obtained (**Figure 6A**), whereas the intact protein was almost not detectable.

3.3.2. Cry1Ab Toxin Digesta Immunoreactivity. The immunoreactivity of the toxin digesta was further analyzed by Western blot using anti-Cry1Ab mAb120 and antitoxin polyclonal antibodies. As demonstrated in **Figure 6B** with the monoclonal antibody, the binding of the toxin decreased as the relative pepsin quantity increased. The same results were obtained when performing Western blot with polyclonal antibodies (data not shown). Nevertheless, none of the degradation products observed in gels were detected either by the monoclonal (**Figure 6B**) or by the polyclonal antibodies used (data not shown).

4. DISCUSSION

Although no clear causal relationship between digestibility and allergenicity is well established, stability to digestion is still considered to be a predictive tool to assess the allergenic potential of a protein (*3*, *8*, *13*). Recently, consideration of SGF test protocol in combination with other physiologically relevant in vitro digestion models and using immunological assays have been proposed to increase the value of stability to digestion data in the overall allergenicity assessment (*34*). The aim of the present work was then to investigate the stability of Cry1Ab insecticidal proteins expressed in GM crops such as MON 810 or other insect-resistant maizes in different conditions of pepsin digestion. Electrophoresis patterns and residual immunoreactivity of digesta were analyzed.

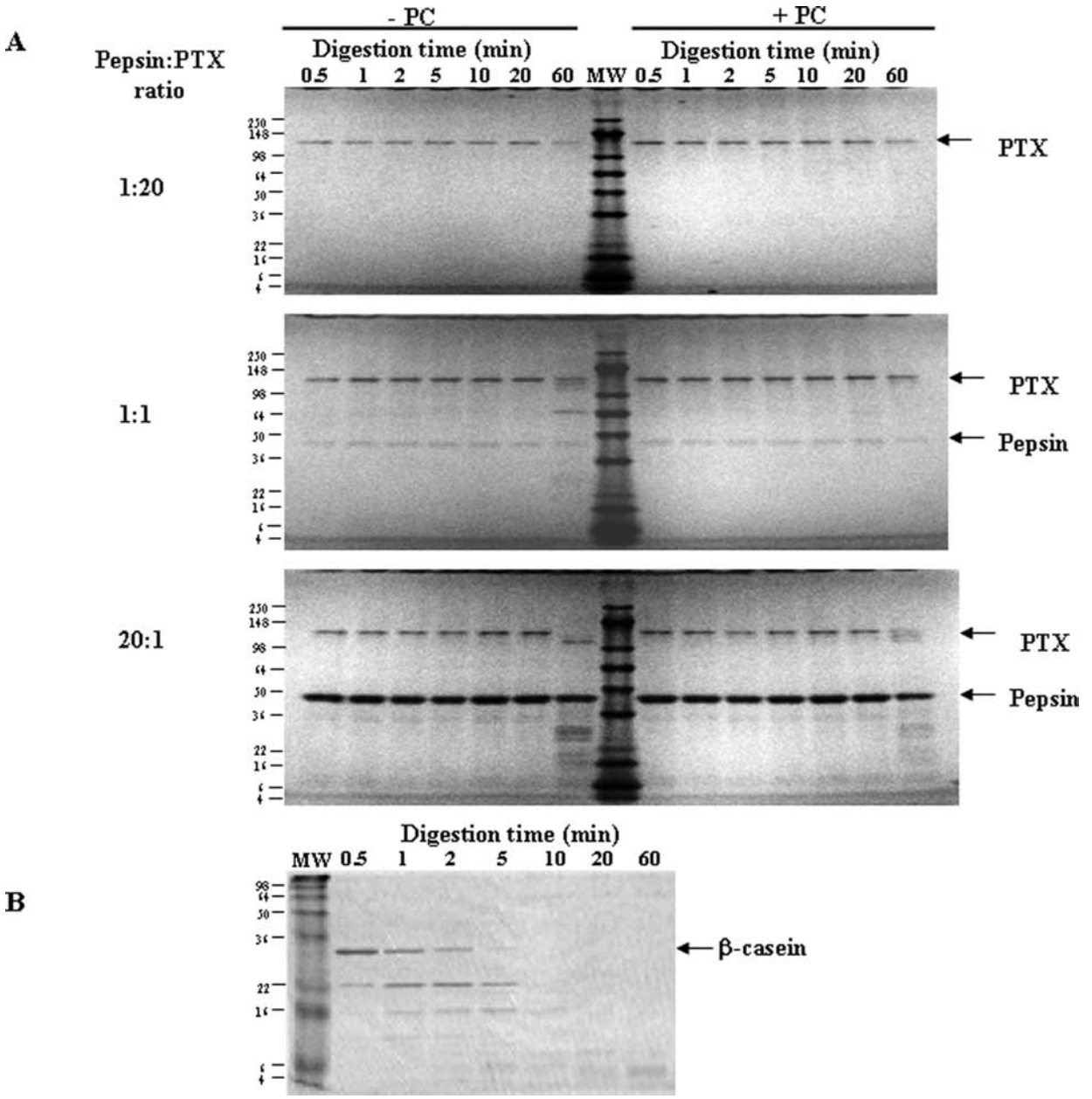

Figure 4. In vitro gastric digestion of Cry1Ab protoxin at pH 2.5: (**A**) 1:20, 1:1, and 20:1 pepsin/Cry1Ab protoxin ratio, without (−) and with (+) PC; (**B**) 1:20 pepsin-to-β-casein ratio without PC. Protoxin, pepsin, and β-casein proteins are indicated. MW, SeeBlue Plus2 Prestained standard marker (Invitrogen). Protoxin (PTX) or β-casein was submitted to in vitro pepsin digestion at pH 2.5. Aliquots were taken at 0.5, 1, 2, 5, 10, 20, and 60 min after the start of gastric digestion and were loaded on 4−20% (PTX) or 15% (β-casein) gels.

As a first step, we checked the in vitro digestibility of Cry1Ab protoxin. We used in vitro gastric digestion models with different pH values and protein-to-enzyme ratios, one of them being more physiologically relevant as based on in vivo data (*14*, *15*). These tests are based on the use of SGF, as recommended by FAO/WHO, Codex Alimentarius, and EFSA, for the assessment of the allergenicity of newly expressed proteins in GMOs (*3*−*5*). Using a "standard" or "high-protease" in vitro digestibility test, we demonstrated that Cry1Ab protoxin is extensively degraded after pepsin treatment at pH 1.2, but only slightly degraded at pH 2.0. Although no difference of pepsin susceptibility was observed for several allergens and nonallergens after adjustment of pH values from 1.2 to 2.0 (*13*, *35*), an increase of the pH from 2.5 to 2.75 completely abrogated the digestion of cod proteins (*36*). An increase in pH from 1.5 to 2.5 has also been demonstrated to significantly reduce pepsin breakdown of kiwifruit allergens (*37*). Altogether, these results demonstrate that slight modification of pH values can significantly modify the persistence of some proteins. Although pH values would influence pepsin activity, porcine pepsin has been demonstrated to be optimally active at pH 2.2, and it is still highly efficient at pH 1.2 (90% activity) and pH 2.5 (70% activity) (*38*). pH can also influence the protein structure, then modifying accessibility to cleavage sites and finally contributing to changes in patterns of proteolysis obtained. Results on digestibility and immunoreactivity obtained with Cry1Ab protoxin at pH 1.2 in the present study corroborate this hypothesis.

In more physiologically relevant conditions (i.e., pH 2.5, pepsin-to-protein ratio of 1:20), Cry1Ab protoxin is not degraded even after 60 min of pepsin digestion. Increasing pepsin quantities (i.e., enzyme-to-protein ratios from 1:1 to 20:1) contributed to the increase of the Cry1Ab protoxin digestion. This is consistent with

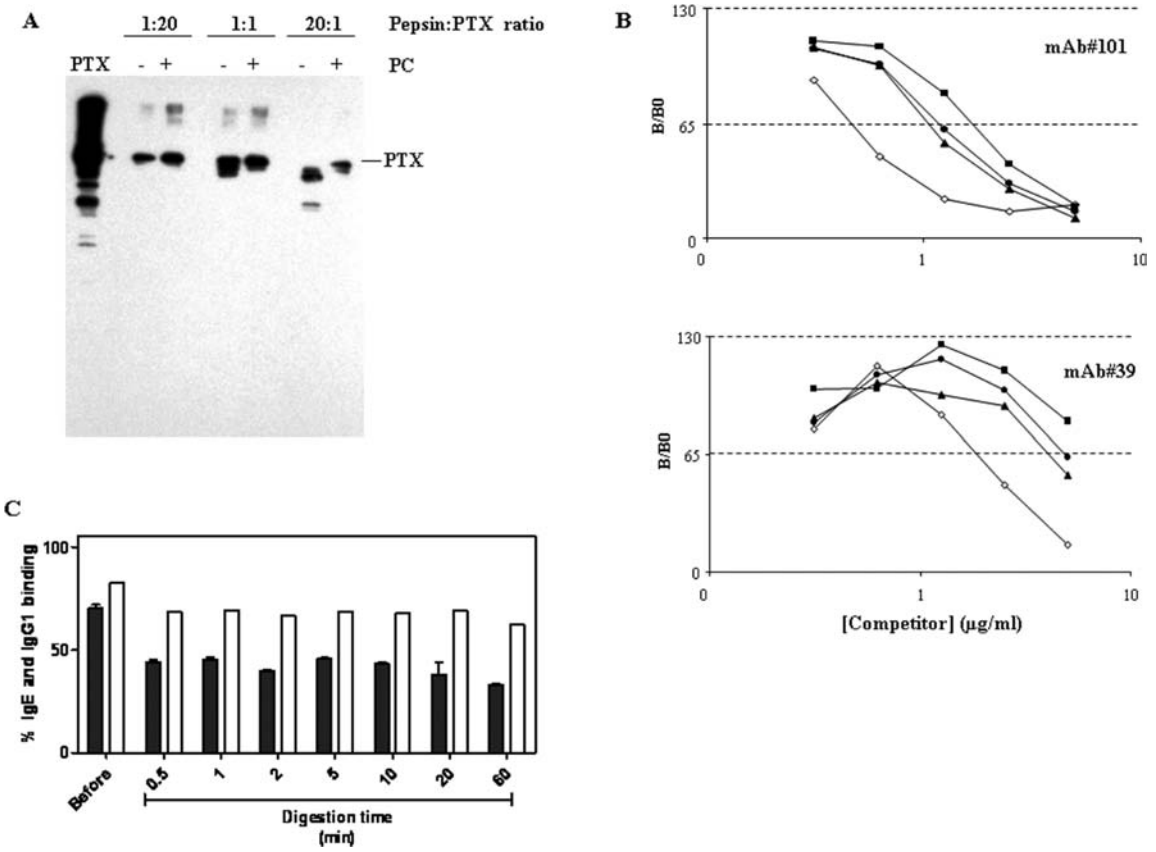

Figure 5. Immunoreactivity of Cry1Ab protoxin digesta obtained after in vitro digestion at pH 2.5. (**A**) Samples obtained after 60 min of pepsin digestion at different pepsin to protein ratios with (+) and without (−) PC, at pH 2.5, were blotted with the anti-Cry1Ab protoxin mAb120. Nondigested purified Cry1Ab protoxin (PTX) is indicated. MW, SeeBlue Plus2 Prestained standard marker (Invitrogen). (**B**) Competitive immunoassays were performed between biotin-labeled protoxin and protoxin before or after 60 min of pepsin digestion at different pepsin to protein ratios without PC: (◇) untreated and nondigested Cry1Ab protoxin; (■) 1:20 pepsin/Cry1Ab digestion ratio; (▲) 1:1 pepsin/Cry1Ab digestion ratio; (●) 20:1 pepsin/Cry1Ab digestion ratio. (**C**) Digesta obtained at different time points from 1:1 ratio without PC were passively immobilized onto microtiter plates. IgE (black bars) and IgG1 (open bars) binding capacities of the different fractions were assayed using pooled sera from mice experimentally sensitized with Cry1Ab protoxin, as explained in **Figure 3**. Reference signal (100%) was obtained using immobilized protoxin not submitted to acidic conditions. "Before" indicates protoxin diluted in SGF pH 2.5 without pepsin.

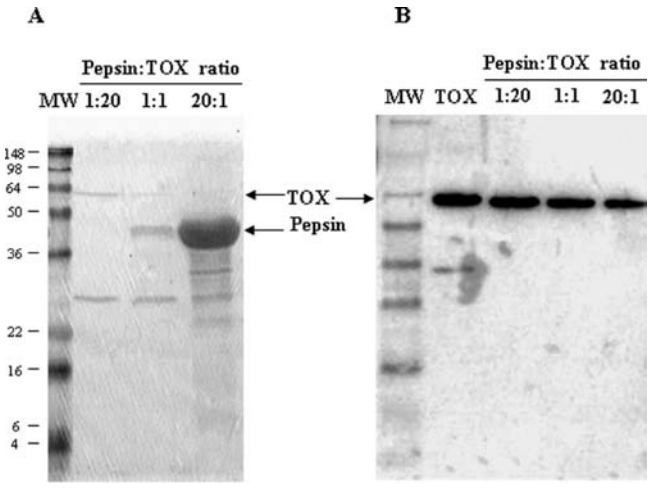

Figure 6. Digestibility and immunoreactivity of Cry1Ab toxin. In vitro digestibility test of Cry1Ab toxin by pepsin at pH 2.5 was performed as for protoxin: (**A**) SDS-PAGE of samples taken after 60 min of digestion at 1:20, 1:1, and 20:1 pepsin/Cry1Ab ratios (without PC); (**B**) immunoblotting of samples obtained after 60 min of pepsin digestion at 1:20, 1:1, and 20:1 pepsin/Cry1Ab ratios without PC using the mAb120. Cry1Ab toxin (TOX) and pepsin are indicated. MW, SeeBlue Plus2 Prestained standard marker (Invitrogen).

the results obtained by Fu and co-workers, demonstrating that increasing the weight ratio of pepsin to test protein from 0.1 to 10 affected the degradation rate of almost all proteins tested except bovine β-lactoglobulin (*12*). β-Casein submitted to the same digestion protocols was rapidly degraded, whatever the pH and the ratio considered. At pH 1.2 and high pepsin-to-protein ratio no intact protein was detectable, as previously described (*7*,*39*,*40*). Digestion was slower using a physiologically relevant model, as intact protein disappeared after 5 min of digestion, which is in line with previous studies (*15*). As modification in pepsin resistance is clearly demonstrated in the presence of phosphatidylcholine, a physiological surfactant secreted by gut mucosa (*7*), we also performed in vitro digestion of Cry1Ab protoxin at pH 2.5 in the presence of PC. We demonstrated that PC protects Cy1Ab from pepsin digestion even at the highest pepsin-to-Cry1Ab ratio, as evidenced by electrophoresis and Western blot analysis.

Additional competitive antibody binding studies demonstrated that the immunoreactivity of protoxin digested for 60 min at pH 2.5 and with low pepsin-to-protein ratio was slightly decreased, which was partially due to the acidic conditions. When using sera from mice experimentally sensitized to the full-length protoxin, we evidenced that IgE and IgG1 binding capacities of Cry1Ab protoxin were highly decreased after digestion at pH 1.2, likely correlating with the extensive degradation of the protein in this digestion model. Conversely, IgE and IgG1 binding capacities were only slightly decreased at pH 2.0 or 2.5. Altogether, these

results demonstrated that Cry1Ab protein structure and immunoreactivity are completely or only slightly altered after gastric digestion, depending on the conditions used in the digestibility test considered.

We then assessed the stability to pepsin of Cry1Ab toxin, corresponding to the N-terminal part of Cry1Ab protoxin expressed in some IR maizes. We demonstrated that although a significant degradation occurred, which was correlated to the quantity of pepsin used, some intact toxin is still present after 60 min of pepsin digestion using a physiologically relevant digestion model (pH 2.5, pepsin-to-protein ratio 1:20). These results were supported by electrophoresis and Western blot analysis.

Our results are in agreement with previous studies demonstrating a rapid degradation of Cry1Ab protoxin and toxin produced in MON 810 or Bt11 maize or expressed as recombinant proteins in bacteria, when using the standard in vitro digestion model (*41*, *42*). Nevertheless, when digestion was performed at pH 2.5 and with the lowest pepsin-to-protein ratio, with or without the addition of surfactants, almost no degradation of Cry1Ab was detected. Considering the latter conditions for in vitro digestibility testing as being more physiologically relevant, as it used a pH value and a pespin-to-protein ratio that are closer to in vivo gastrointestinal conditions, our results suggest that a fraction of the protoxin and, to a lesser extent, toxin could reach the duodenum as an intact protein. In vitro trypsin digestion of Cry1Ab protoxin with different enzyme-to-Cry1Ab molecular ratios (from 50:1 to 0.5:1) resulted in degradation of the protein. However, resulting fragments,which ranged from 43 to 74 kDa, were still present after 24 h of digestion (*43*). This suggests that intact Cry1Ab remaining after gastric digestion will be only partially degraded in the duodenum. All of those results are in accordance with in vivo data on different animal species. In calves and pigs, Cry1Ab toxin from Bt11 maize was recovered in different parts of the gastrointestinal tract, but no transfer to liver, spleen, or lymph nodes was evidenced (*44*, *45*). Bt176 maize digestion in cow's rumen for up to 48 h demonstrated the progressive cleavage of Cry1Ab toxin, concomitant with the appearance and persistence of 17 and 34 kDa fragments (*46*). The same authors also evidenced that the whole protein was still present after 8 h of digestion, and the 17 kDa band was detectable in bovine feces, demonstrating the high resistance to digestion (*47*). Altogether, these studies and our results suggest that Cry1Ab digestion resistant fragments of at least 17 kDa could reach the mucosal immune system. However, due to the low amount of Cry proteins expressed in insect-resistant GM maizes approved so far, and the limited exposure of human consumers to foods derived from GM maizes, it is unlikely that such low doses of Cry1Ab fragments would induce a mucosal immune response in humans.

In conclusion, in the context of allergenicity assessment of GM foods, digestibility of newly expressed proteins should be further analyzed using both standard pepsin-resistant test and more physiologically relevant in vitro gastrointestinal digestion models reflecting the conditions of human digestion in the general population or even at-risk groups with modified or impaired digestive function. In addition, testing the purified protein both in solution and in the whole complex food taking into consideration the interactions with the food matrix that may alter the digestion would certainly improve the weight-of-evidence approach used to assess the allergenicity of novel proteins.

ABBREVIATIONS USED

SGF, simulated gastric fluid; PC, phosphatidylcholine; GM, genetically modified; IR, insect resistant; EIA, enzyme immunoassays; mAb, monoclonal antibodies; sSGF, standard simulated gastric fluid; pSGF, "physiological" simulated gastric fluid; AChE, acetylcholinesterase.

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