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Rapid Digestion of Cry34Ab1 and Cry35Ab1 in Simulated Gastric Fluid

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Two genes were identified in *Bacillus thuringiensis* Berliner (Bt) that code for the proteins that comprise a Cry34Ab1/Cry35Ab1 binary insecticidal crystal protein. Maize, *Zea mays* L., plants have been transformed to express the Cry34Ab1/Cry35Ab1 proteins, and as a result, these plants are resistant to attack by western corn rootworm, *Diabrotica virgifera virgifera* LeConte, a major pest in the Midwestern corn-growing area of the U.S.A. As part of the safety assessment for the proteins, digestibility studies were conducted. Digestion experiments with both proteins demonstrated rapid degradation in simulated gastric fluid, comparable to other registered plant-incorporated protectants. Quantitative and qualitative approaches for determining digestibility are illustrated.

INTRODUCTION

Two genes were identified in *Bacillus thuringiensis* Berliner (Bt) that code for the proteins that comprise a Cry34Ab1/Cry35Ab1 binary insecticidal crystal protein (ICP) (*1*). Maize, *Zea mays* L., plants have been transformed to express the Cry34Ab1/Cry35Ab1 proteins, and as a result, these plants are resistant to attack by western corn rootworm, *Diabrotica virgifera virgifera* LeConte, a major pest in the Midwestern corn-growing area of the U.S.A. (*2*, *3*). The Cry34Ab1 protein is approximately 14 kDa in size, and the Cry35Ab1 protein is approximately 44 kDa in size.

As part of the effort to evaluate the safety of plant-incorporated protectants, i.e., ICPs that are expressed in crops, the digestibility of the proteins in simulated gastric fluid (SGF) was investigated. Although somewhat controversial (*4*), some have suggested a correlation between the in vitro stability of a protein in SGF and the ability of the protein to be an allergen (*5*). A recent study suggests that this correlation is not likely the result of pepsin-mediated digestion in the stomach but rather may indicate a biochemical property of the protein that predisposes it to intracellular digestion (*6*). Thus, in vitro digestibility experiments are designed to measure a biochemical property of the tested proteins that may correlate with allergenicity but are not necessarily intended to simulate in vivo digestion (*7*). More complex in vitro models are employed to mimic in vivo digestion (*8*).

Digestibility evaluations have typically been accomplished by exposing the proteins of interest to SGF for various periods of time and then assessing when the proteins (or breakdown fragments) are no longer detectable on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) and/or western blots (*9*−*12*). When combined with a measurement of the sensitivity to visualize protein bands on these gels, this technique is useful for determining an upper limit of point estimates of decay, such as half-life. Here, we report on the SGF digestibility of Cry34Ab1 using a more quantitative approach based on classical enzyme theory. We also report results for Cry35Ab1 using the aforementioned qualitative approach for determining the upper limit of point estimates of decay.

Previous digestibility studies with transgenic ICPs were largely qualitative and were based on the lack of detection on SDS−PAGE gels and western blots after exposure to SGF for a specific period of time (*9*−*12*). As such, they determined the length of time it took for the ICP to degrade below the sensitivity of the analytical technique. When the sensitivity of the analytical technique is known, it is possible to conclude when the ICP was degraded below the established assay sensitivity. For example, if a protein is loaded on a gel at 200 ng/lane and as little as 20 ng can be detected on the gel, then at least 90% degradation has occurred for any sample that is not visible on the gel. If the protein can be seen on the gel after 1 min of exposure to SGF, but not after 2 min, then it can be concluded that the DT_{90} (time until 90% decay) is less than 2 min. Here, we report results for Cry35Ab1 using this approach.

A more quantitative approach to measuring digestion rate is to quantify the residue after exposure to SGF for various time intervals and to model the decay. Quantification of Bt proteins based on the density of the bands on SDS−PAGE gels has previously been described (*13*). Classical enzyme theory can be used to model the degradation. The Michaelis−Menten rate law describes enzymatic reactions. When the enzyme is present at a substantially higher concentration than the substrate, as has been the typical case for ICP digestibility studies, the degradation is usually well-described by first-order kinetics (*14*). This simple model can be used to calculate point estimates of decay such as a half-life or DT_{90}. For decay that follows first-order

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kinetics, point estimates describe the decay rate over the entire degradation profile.

METHODS AND MATERIALS

Cry34Ab1 and Cry35Ab1 protein preparations were individually purified from two different recombinant *Pseudomonas fluorescens* strains, each engineered to produce a single component of the binary ICP (*2*). Cry34Ab1 was purified as a 54% active ingredient (AI) powder, and Cry35Ab1 was purified as a 37% AI powder. Microbe-produced proteins were used for this work due to the difficulty in isolating plant-derived proteins. The equivalency of the microbe-produced and plant-produced proteins has been established biochemically and functionally (*15*).

SGF containing 0.32% (w/v) pepsin (Sigma Aldrich, St. Louis, MO) was prepared at pH 1.2 as described in the United States Pharmacopoeia (*16*). Solutions of the proteins were diluted 20-fold with SGF to produce initial concentrations of Cry34Ab1 and Cry35Ab1 of ~27 and 61 μg AI/mL, respectively. These concentrations resulted in ICP:pepsin molar ratios of ~1:45 and 1:62 for Cry34Ab1 and Cry35Ab1, respectively, and were chosen to achieve a high ratio of pepsin to substrate. This is consistent with other Bt digestion studies and thus facilitates comparisons among Bt proteins. The predominance of enzyme as compared to substrate also allows the decay to be modeled using first-order kinetics.

Vessels of SGF were placed in a 37 °C water bath for 3−5 min, followed by the addition of protein solutions. The vessels were gently agitated during the digestions. Aliquots of the digestion stock were removed at the desired incubation intervals and neutralized with sodium carbonate to stop the reaction (*10*). Solutions for zero time points were prepared for the qualitative studies by substituting water for SGF and were prepared for the quantitative studies using neutralized SGF. SGF blanks were prepared by substituting water for protein. Samples were stored on ice or frozen until analysis. The digestion experiment with the Cry34Ab1 was performed twice to assess the consistency of results.

Analyses by SDS−PAGE and western blotting were accomplished as follows. The protein digestion samples were mixed with equal volumes of Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) containing freshly added β-mercaptoethanol (5% v/v, Sigma Aldrich). Polyacrylamide (4−20%) gels (Zaxis Laboratories, Hudson, OH, or Owl Separation Systems, Portsmouth, NH) were loaded with approximately 0.19 μg of Cry34Ab1 and 0.61 μg of Cry35Ab1 per lane (based on predigestion levels). The samples were electrophoresed through the gel at 125−150 V for 2−3 h. A single gel was run for the qualitative assays, and triplicate gels were run for the quantitative assays. Gels were stained with Coomassie Brilliant Blue dye (Pierce GelCode Blue or Invitrogen G250) and destained until the background was clear.

For Cry35Ab1, an additional gel was prepared to determine the limit of detection for this protein by SDS−PAGE analysis. A 2-fold serial dilution series was loaded on the gel, and the volumes of the bands were determined by densitometry using the same methods as described later in this section for Cry34Ab1.

To positively identify the ICPs on the gels, an additional gel was prepared for each protein for use in western blot analyses. For these gels, the proteins were diluted an additional 10-fold for Cry34Ab1 protein or 40-fold for the Cry35Ab1 protein, prior to loading. Proteins on these gels were transferred to nitrocellulose membranes using a Muliphor II NovoBlot Electrophoretic Apparatus or a Bio-Rad Criterion Blotter. Following protein transfer, the membranes were blocked with Tris buffered saline containing 0.3% Tween 20 and 5% powdered milk. Polyclonal antibodies specific to the individual proteins were then added to the appropriate blots to allow for protein detection. The antibodies were raised to highly purified microbe-produced proteins. Following an incubation period, the membranes were washed, and a secondary antibody conjugated to horseradish peroxidase (Bio-Rad Laboratories) was added. After another incubation period, membranes were washed again, and a chemiluminescent solution (Amersham Pharmacia Biotech, Piscataway, NJ) was added. Detection film was then exposed to the blots and developed with a Konica SR-X film developer.

Quantification of the Cry34Ab1 on SDS−PAGE gels was accomplished by measuring the density of the appropriate bands. After they were destained, the gels were placed on a glass platter of a densitometer (Molecular Dynamics model PD-120, Sunnyvale, CA), and each gel was scanned using a 100 μm and 12 bit setting. This provided the desired level of detail when the protein bands were viewed under 4× magnification. Quantification was achieved by using rectangular volume integration (optical density × area) of the individual Cry34Ab1 bands. The densitometric quantification was expressed as peak volume per band. After correcting for background, the peak volume was directly proportional to the quantity of protein that was present (*13*).

The decay rate of the Cry34Ab1 protein was fit to a first-order model, and the half-life and DT_{90} were estimated from the model. A first-order decay model was determined by regressing the natural logarithm of the percent remaining (based on SDS−PAGE densitometry estimates) against time. The volume at each time point was determined for each gel by subtracting the background, as determined for that gel, from the total volume, to arrive at a corrected volume. The average 10, 15, and 20 min digestion volumes were used as the background because the volumes at these time points did not consistently decrease with time, although the more sensitive western blot analysis demonstrated that decay continued over this period. These volumes were also consistent with the SGF only volumes but provided more realistic background numbers since they contained all components present in the actual digestions with the exception of the intact Cry34Ab1 protein. Autodegradation fragments from the pepsin appeared to be substantial contributors to the background volumes, but their abundance appeared to vary with time (**Figure 3**) and were also likely mediated by competition with the Cry34Ab1 protein. Thus, the later degradation time points, where the measured volumes stabilized, were considered to be the best estimate of background. Percent remaining Cry34Ab1 was determined on each gel by dividing the corrected volumes at each time point by the corrected volume at the zero time point and multiplying by 100. The average percent remaining across the gels was used to fit a first-order decay model. The half-life and DT_{90} were determined by substituting the natural logarithm of 50 and 10% of the intercept, respectively, into the regression equation. The 0−7.5 min time points were modeled.

The first-order regression equations generated from the SDS−PAGE densitometry volumes were also used to calculate the sensitivity of the Cry34Ab1 western blot. This was accomplished by substituting the last time point where the Cry34Ab1 was visible on the western blot into the regression equations and solving for the percent remaining. The least sensitive estimate is reported.

RESULTS AND DISCUSSION

Both Cry34Ab1 and Cry35Ab1 were seen to be digested in SGF based on the decrease in intensity and disappearance on SDS−PAGE gels and western blots (**Figures 1**−**3**). Western blots were also useful for confirming the identity of the proteins and their degradation fragments. The limit of detection for Cry35Ab1 by SDS−PAGE analysis was found to be <15.6 ng/lane based on results from a gel containing a serial dilution series of the Cry35Ab1 protein (**Figure 4**). Because approximately 0.61 μg/lane of Cry35Ab1 was loaded on the SDS−PAGE gel (based on undigested quantity), the analytical sensitivity of the assay was <2.6% of the amount present before digestion. Therefore, when the Cry35Ab1 was no longer visible on the SDS−PAGE gel after 5 min (**Figure 1**), greater than 97% of the protein had been digested. Because these data were consistent with rapid digestion and with the profile of other registered plant-incorporated protectants (*7*), more quantitative studies were not undertaken with this protein. **Figure 1** shows that after 1 min of digestion, the Cry35Ab1 was primarily present as smaller molecular mass fragments of approximately 40 and 15 kDa, but these fragments were not detectable after 5 min of digestion.

By SDS−PAGE and western blot analysis, Cry34Ab1 was visible on gels and blots after 15 min but not after 20 min of exposure to SGF (**Figures 2** and **3**). Western blots also showed

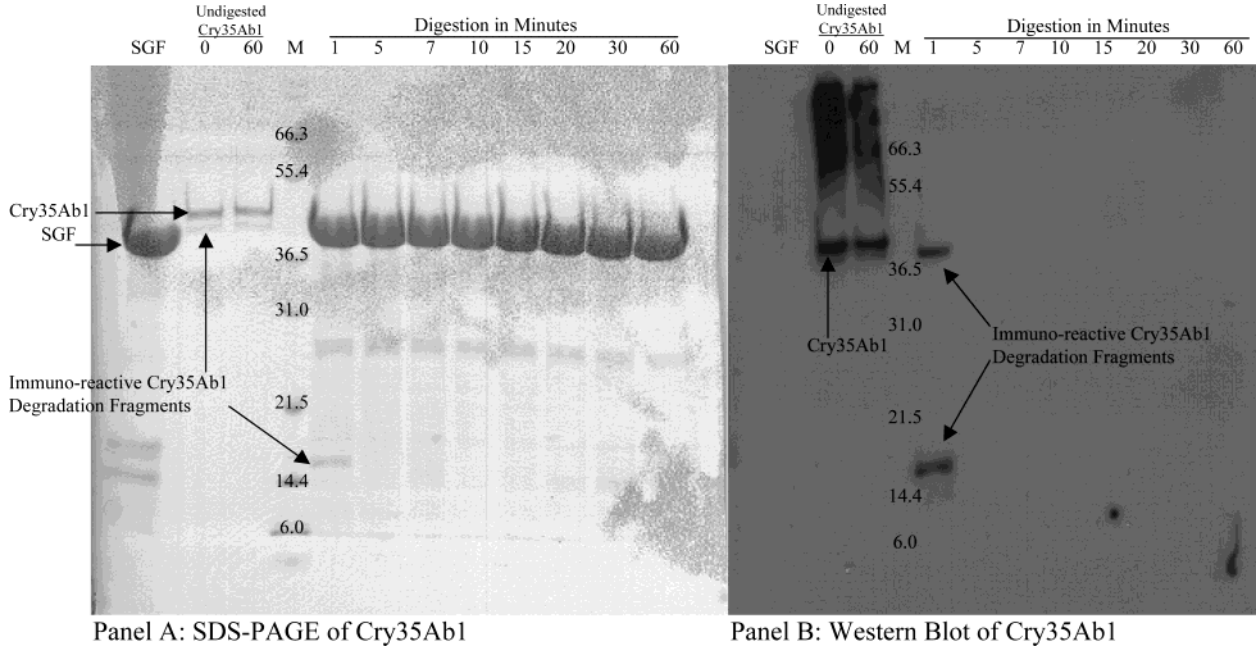

Figure 1. SDS–PAGE and western blot of Cry35Ab1 SGF digestion. M = molecular mass markers in kilodaltons. The SDS–PAGE gel was loaded at 610 ng AI/lane (predigestion) and the western blot was loaded at 15 ng AI/lane (predigestion).

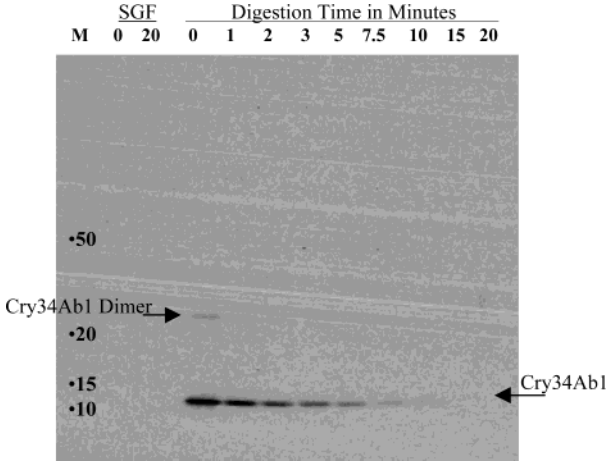

Figure 2. Western blot of Cry34Ab1 SGF digestion. M = molecular mass markers in kilodaltons. Blot loaded at 19 ng AI/lane (predigestion).

a small amount of dimer to be present for digestion intervals of less than 7.5 min (demonstrated by size exclusion chromatography; not reported here). No breakdown fragments were observed.

On the basis of the first-order decay demonstrated by the digestion, we calculated that the sensitivity of our western blot detection methods to the target protein was <0.54% remaining (**Figures 2** and **5**). As a result, our detection techniques appear to be more sensitive than those used for previous studies (*11*). Therefore, we could detect residual Cry34Ab1 that may not have been detectable at the sensitivity reported for some previous digestion studies conducted with Bt proteins (~10% of target protein remaining, *11*). To generate comparable data, a less sensitive detection method or a more quantitative approach was needed. Because our study, as well as previous studies with other Bt proteins, all appeared to maintain a high pepsin-to-protein ratio, it was possible to use first-order kinetics based on a simple derivation of the Michaelis−Menten rate law to generate point estimates of digestion (*14*); these results could be compared with existing data for other Bt-based plant-incorporated protectants.

Protein quantification based on the density of bands on SDS−PAGE gels had previously been reported (*13*), so we could quantify the residues on the SDS−PAGE and model the results. The density of bands seen on the SDS−PAGE gel for the Cry35Ab1 sensitivity analysis also confirmed the direct relationship between band density and protein quantity, except at low protein concentrations where the density may overestimate the actual amount of protein present (**Figure 4** and **Table 1**). This provided additional support for excluding low volumes (later time points) from our kinetic analyses.

Cry34Ab1 degradation followed classical first-order kinetics and was highly reproducible (**Figure 5**). Estimated half-lives for the two experiments were 1.9 and 2.0 min, and DT_{90} values were estimated at 6.3 and 6.8 min. DT_{90} values were calculated because this was the limit of detection in digestion experiments with some Bt proteins expressed in currently registered plant-incorporated protectants (*11*) and thus provided a means for comparing the digestibility of the Cry34Ab1 protein to these proteins. The Bt proteins expressed in all currently registered plant-incorporated protectants become undetectable at or below 7 min (*7*). The current kinetic analysis allowed the digestion experiments conducted with Cry34Ab1, using highly sensitive analytical methods, to be compared directly to previous studies and demonstrated that the digestibility of the Cry34Ab1 protein is consistent with the rapid digestion reported for registered Bt proteins (DT_{90} < 7 min).

For qualitative digestion experiments, it is recommended that the sensitivity of the analytical technique be reported along with the time when the protein of interest is no longer visible on gels or blots. This allows an upper limit for a specified decay point estimate to be reported. For example, the DT_{97} for Cry35Ab1 as determined here is less than 5 min.

It is also important for both quantitative and qualitative studies to ensure that a significant molar excess of pepsin is present in the digestion experiments, so first-order kinetics will likely describe the decay pattern. Point estimates of decay, such as half-lives and DT_{90} values, are often independent of substrate concentration only at low protein-to-enzyme ratios. If this is not the case and a mixed-order reaction is observed, then

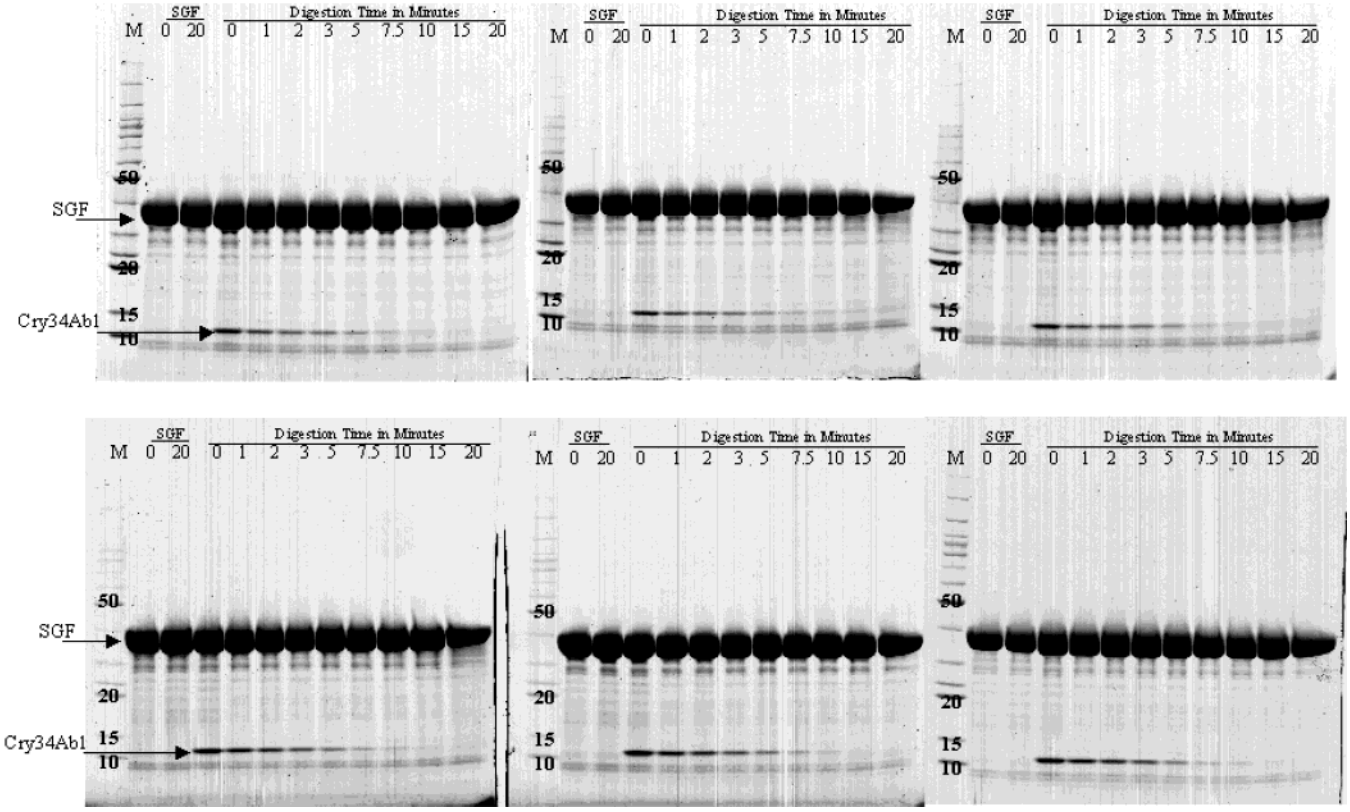

Figure 3. SDS–PAGE gels from Cry34Ab1 digestions. The gel was loaded at 190 ng AI/lane (predigestion). Each row represents a separate experiment (three replicate gels). Note the light background bands at approximately 14 kDa in lanes containing SGF only that are of similar intensity to those seen at later Cry34Ab1 digestion times.

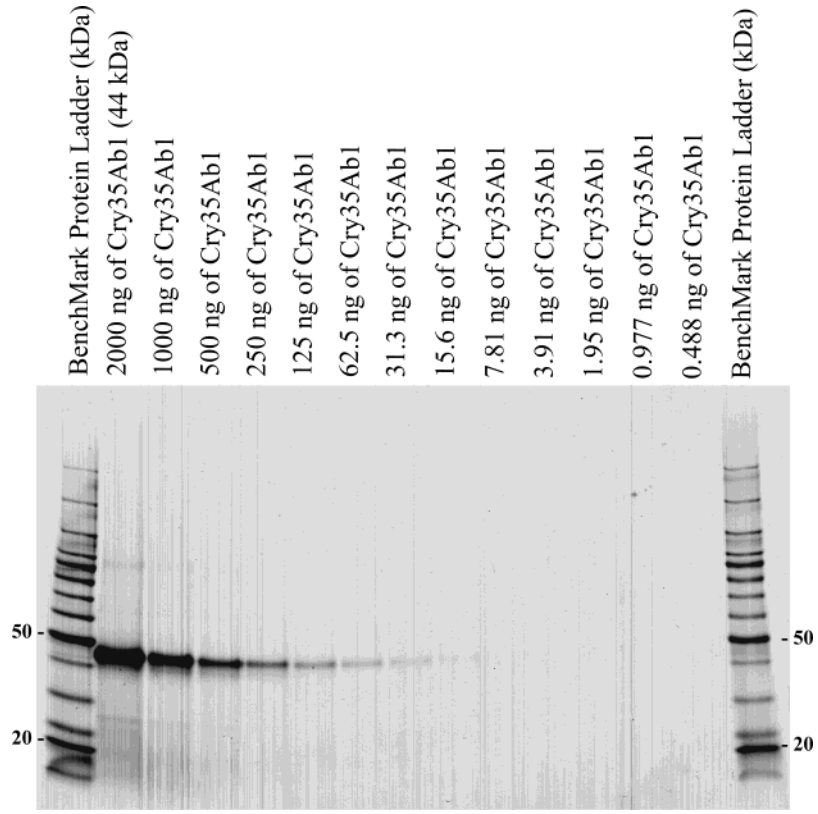

Figure 4. SDS–PAGE gel of a 2-fold serial dilution of Cry35Ab1.

comparisons among proteins will be far more difficult, because digestion rates will not vary in a uniform manner in relation to the substrate concentration. Thus, at high substrate-to-enzyme ratios, point estimates of decay will often be dependent on the initial protein concentration, making comparisons among proteins difficult. This is further complicated by the varying sensitivity of analytical techniques for detecting different proteins.

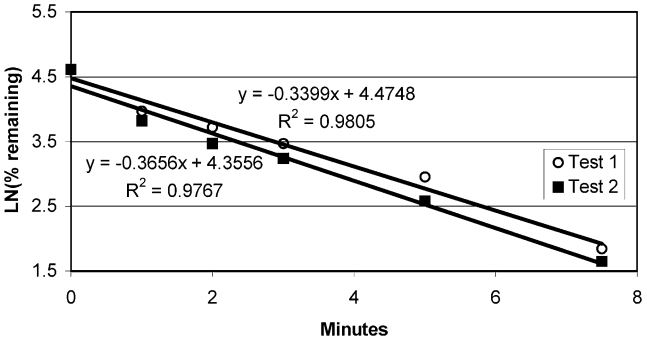

Figure 5. Comparison of Cry34Ab1 digestion between two experiments.

Table 1. SDS–PAGE Densitometry Volumes, Expected Volumes Based on 2000 ng Loading, and Residuals (Percent Difference between Observed and Predicted Value)[a]

Cry35Ab1/ lane (ng)	measured volume	expected volume	% residuals
15.6	7.40	4.22	75.2
31.3	16.12	8.45	90.9
62.5	24.04	16.89	42.3
125.0	33.38	33.78	–1.2
250.0	62.76	67.56	–7.1
500.0	140.70	135.13	4.1
1000.0	294.10	270.25	8.8
2000.0	540.50	540.50	0.0

[a] Linear regression equation of measured volumes vs Cry35Ab1 amounts between 125 and 2000 ng/lane; $y = 0.2731x + 2.6392$, $R^2 = 0.9972$.

It is acknowledged that not all enzymatic reactions behave according to Michaelis−Menten kinetics and that very pepsin resistant proteins may not exhibit first-order kinetics, even when present at much lower concentrations than pepsin. However, these exceptions will be readily apparent during a kinetic analysis. In the latter case, the qualitative determination that the protein is very pepsin resistant may answer the investigators question. In addition to the intact protein substrate, degradation fragments may be observed to accumulate and degrade over time. Approaches to modeling the decay rates for such fragments have been established using first-order kinetics (*17*).

For previous SGF digestion studies with Bt proteins, the pepsin concentration has typically been held constant at ~0.32%. Thus, we recommend that the molar ratio between pepsin and the protein of interest be adjusted through the selection of an appropriate protein concentration. Using the quantitative approach described here, or qualitative results with sensitivity limits, it is possible to meaningfully compare SGF digestion across experiments where different levels of analytical sensitivity may have existed.

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