

Quantitative measurement of protein digestion in simulated gastric fluid

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Abstract

The digestibility of novel proteins in simulated gastric fluid is considered to be an indicator of reduced risk of allergenic potential in food, and estimates of digestibility for transgenic proteins expressed in crops are required for making a human-health risk assessment by regulatory authorities. The estimation of first-order rate constants for digestion under conditions of low substrate concentration was explored for two protein substrates (azocoll and DQ-ovalbumin). Data conformed to first-order kinetics, and half-lives were relatively insensitive to significant variations in both substrate and pepsin concentration when high purity pepsin preparations were used. Estimation of digestion efficiency using densitometric measurements of relative protein concentration based on SDS-PAGE corroborated digestion estimates based on measurements of dye or fluorescence release from the labeled substrates. The suitability of first-order rate constants for estimating the efficiency of the pepsin digestion of novel proteins is discussed. Results further support a kinetic approach as appropriate for comparing the digestibility of proteins in simulated gastric fluid.

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1. Introduction

Proteins that may have been present only rarely in food are being incorporated into crops via molecular biological techniques (Mendelsohn et al., 2003). As part of the human health safety assessment for these proteins, a number of properties are evaluated to assess the likelihood that the protein will be able to induce an allergic response when ingested. The source of the protein is investigated to determine if it has a history of causing allergy, and the amino acid sequence of the protein is compared to that of known allergens to see if there are relevant similarities. Also, the prevalence of the protein in the transgenic crop and the stability of the protein to heat and digestive conditions are examined (Codex, 2003; Metcalf et al., 1996).

Proteins found to be labile in simulated gastric fluid (SGF) are believed to represent a reduced risk of inducing food allergy due to their instability after ingestion (Astwood et al., 1996). While in vitro SGF methods do not reproduce in vivo gastric conditions (Mendelsohn et al., 2003), results from such assays have sometimes (Astwood et al., 1996), but not always (Fu et al., 2002), been correlated with allergenic potential. The association between digestibility in SGF and food allergy may be due to a correlation between in vivo and in vitro gastric digestion, or may represent a correlation between susceptibility to pepsin and susceptibility to intracellular proteases (Dearman et al., 2002). In either case, the result would be a reduced titer of protein available for systemic absorption and subsequent interaction with IgE antibodies.

Previously, quantitative approaches for comparing the digestion of different proteins in SGF have been described (Herman et al., 2003). Here, we report additional

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results from experiments designed to evaluate the validity of a kinetic approach to quantifying digestion, and to determine the appropriate method for standardizing pepsin preparations. Specifically, the fit of digestion data to a first-order kinetic model was investigated under low substrate and high enzyme conditions, and the effect of normalizing pepsin preparations based on specific activity in a standard hemoglobin assay (Anson, 1938) was compared to normalization based on the purity of the pepsin preparations. Chromogenic and fluorogenic protease substrates were used to allow digestion to be tracked via spectrophotometric or fluorometric measurements (absorbance or fluorescence). These substrates release dye or fluoresce as they degrade, facilitating the quantitative measurement of digestion. Results from the spectrophotometric or fluorescence measurements were also compared to density measurements of substrate bands on SDS–PAGE gels to determine if different methods of quantifying intact protein yield similar estimates of digestibility.

2. Methods

2.1. Digestions

Five pepsin lots of differing purity and activity were obtained from Sigma Aldrich (St. Louis, MO—Cat. #P-6887, 86% purity, 4230 U of activity/mg protein; Cat. #P6887, 100% purity, 3460 U of activity/mg protein; Cat. #P-6887, 90% purity, 3640 U of activity/mg protein; Cat. #P-7012, 76% purity, 2860 U of activity/mg protein; and Cat. #P-7000, 32% purity, 2080 U of activity/mg protein). SGF was prepared with each lot according to standard procedures. (The United States Pharmacopeia 24, 2000, Astwood et al., 1996—0.32% pepsin concentration, pH 1.2, constant mixing.) Additional conditions were explored for the 32, 76, 90, and 100% purity lots of pepsin by normalizing the specific activity of the enzyme to the level of the 86% purity pepsin lot. This normalization resulted in pepsin concentration that ranged from 0.37 to 0.65% for the various pepsin lots. Three digestions of azocoll in SGF containing 0.288% of the 86% purity pepsin were also carried out.

Azocoll (chromogenic collagen—Sigma Aldrich, Cat. #A-4341) and DQ-ovalbumin (fluorogenic ovalbumin—Molecular Probes, Cat. #D-12053, Eugene, OR) suspensions were diluted 20-fold with SGF to produce initial concentrations of 500 and 50 µg/mL, respectively. Concentrations were chosen based on probe experiments designed to determine the linear range of the detection methods (unpublished). Vessels of SGF were placed in a 37 °C water bath on magnetic stir plates and gently mixed for 5 min, followed by the addition of protein solutions. One-milliliter aliquots of the azocoll digestion

mixture were removed at the desired incubation intervals of 0.25, 0.5, 1, 2, 5, 10, 15, 20, 30, 45, and 60 min. One hundred microliters aliquots of the DQ-ovalbumin digestion mixture were removed at 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, and 60 min. The digestion was immediately terminated for each sample by neutralizing the SGF with 400 µL (azocoll) or 40 µL (DQ-ovalbumin) of 200 mM Na₂CO₃, pH 11. The proteins were added to neutralized SGF to produce the initial sample at time zero. Azocoll protein digestion samples were quantitated on a UV-1601 spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD) at a wavelength of 520 nm (single subsample per digestion duration). The DQ-ovalbumin protein digestion samples were diluted and quantitated on a Cary Eclipse fluorometer (Varian Analytical Instruments, Walnut Creek, CA) using an excitation wavelength of 505 nm and emission wavelength of 515 nm (eight subsamples per digestion duration). A minimum of two digestions were completed for each protein with each of the five pepsin lots. A single digestion of DQ-ovalbumin at half the concentration described above (25 µg/mL) was completed using the 86% purity pepsin to evaluate the appropriateness of the first-order model.

2.2. Azocoll SDS–PAGE analysis

SDS–PAGE analysis was carried out by mixing an equal volume of each digestion sample with Laemmli buffer (Bio-Rad Cat. #161-0737) containing 5% 2-mercaptoethanol (Bio-Rad Cat. #161-0710) and heated for 10 min at 100 °C. The supernatant was collected by centrifugation for 1 min at 20,000g. The resulting soluble protein fractions were applied directly to the SDS–PAGE gel for total protein visualization. SDS–PAGE was performed in triplicate using 4–20% Bio-Rad pre-cast gels (Cat. #345-0033) fitted into a Bio-Rad Criterion Gel module. Twenty-five microliters of each sample (various digestion intervals) was loaded per well and the digested protein mixture was separated in the polyacrylamide gel at a constant voltage of 250 V for approximately 1 h using Tris/glycine/SDS buffer (Bio-Rad Cat. #161-0732). After separation, the gel was washed extensively with water and the proteins were visualized with Coomassie brilliant blue dye (Pierce GelCode Blue, Cat. #24592). After the gels were stained for 1 h and destained with water, each gel was scanned with a Molecular Dynamics PD-SI densitometer. The azocoll bands were quantified for each time point using rectangular objects and the volume (optical density × area) was integrated using the instruments default settings.

2.3. Azocoll kinetic analysis

A first-order model (three-parameter exponential) was fit to time versus absorbance data using non-linear

regression ($\text{absorbance} = X - I \times e^{-K \times \text{time}}$). Data for evaluating the fit of digestion data to a first-order model were plotted in the natural scale to illustrate the effect of varying the initial protein substrate concentration (varied due to non-homogeneity of the azocoll suspensions). Data for comparing the various pepsin preparations were normalized for plotting based on setting the intercept at time zero to 100% remaining and the asymptote to zero ($\% \text{ remaining} = 100 \times (X - \text{absorbance})/I$). The latter approach was taken to compensate for potentially different starting substrate concentrations that may have been present between replicate experiments due to lack of homogeneity within suspensions of the azocoll. This method of plotting is also consistent with conventional illustrations of decay, and thus aids in the interpretation of results. A first-order model (three-parameter exponential) was also fit to the sum of the densities of the substrate bands from the SDS-PAGE gels using non-linear regression. The density of the substrate bands in neutralized SGF was not used as zero-time data in the SDS-PAGE analysis due to variability introduced by a separate pipetting of the substrate suspension, but rather the data from 15 s to 60 min was modeled. This was not a factor for the former analysis, since the absorbance in neutralized SGF was only used to estimate the background level of soluble dye. Half-lives were calculated by dividing the natural logarithm of 0.5 by the first-order rate constant.

2.4. DQ-ovalbumin kinetic analysis

A three-parameter exponential equation was also fit to the DQ-ovalbumin data, and fluorescence data were also normalized for plotting based on setting the intercept at time zero to 100% remaining and the asymptote to zero. Data between 5 and 60 min were modeled. An initial rapid release of fluorescence was observed in preliminary experiments (unpublished) that was not consistent with first-order decay so the initial fluorescence in neutralized SGF was excluded from the analysis. This initial release of fluorescence may have been the result of a rapid digestion of an accessible and heavily labeled peptide fragment, or more likely, the digestion of a subset of protein that was denatured or reduced and therefore more accessible to the enzyme (Takagi et al., 2003). DQ-ovalbumin bound Coomassie dye very poorly, possibly due to interference by the bound fluorophore, so SDS-PAGE gels were not useful for tracking the labeled substrate (unpublished). However, quantitative results for non-labeled ovalbumin digestion in SGF were available (Takagi et al., 2003), and these results were modeled using first-order kinetics (three-parameter exponential) for comparison to the previously described experiments.

3. Results and discussion

3.1. Background

Enzymatic reactions often behave according to the Michaelis–Menten rate law. Two parameters (V_{max} and K_m) are typically estimated for enzymatic reactions that together can be used to estimate the efficiency of the reaction (V_{max}/K_m) (Gulledge and Schimel, 1998; Mune and White, 1996; Poet et al., 2003). V_{max} is the maximum velocity of the reaction, and K_m is the concentration of the substrate that results in a reaction that initially proceeds at 50% of V_{max} . It should be noted that, although protein digestion is a multi-step process, one or a small number of rate-determining steps usually determine the kinetics of multi-step reactions (Kluger, 1900).

Under conditions of low substrate concentration (substrate concentration $\ll K_m$), Michaelis–Menten reactions are well described by first-order kinetics (Schnell and Maini, 2000; Schnell and Mendoza, 2004; Tzafiriri, 2003). Rate constants determined under such conditions are often referred to as pseudo-first-order rate constants because the reaction is normally sensitive to changes in both substrate and enzyme concentration. However, if the enzyme concentration remains constant (as for pepsin in the SGF assay), the reaction velocity only varies in response to changes in the substrate concentration (under constant temperature, pH, etc.) and the kinetics appear to be first-order. The first-order rate constant determined from a substrate concentration–time profile generated under such conditions is equal to V_{max}/K_m and thus is a well recognized measure of the efficiency of an enzymatic reaction (equivalent to the pharmacokinetic term “intrinsic clearance”). It is noteworthy that the relationship between the first-order rate constant and V_{max}/K_m should be valid at high enzyme concentrations where V_{max} has reached a plateau. This relationship is most commonly noted for concentrations where V_{max} is directly proportional to enzyme concentration. However, at the minimum enzyme concentration where V_{max} becomes insensitive to further increases in enzyme concentration, the K_m continues to be a constant for the reaction, the V_{max} ceases to increase, and the first-order rate constant for the reaction is stable. Therefore, further increases in the enzyme concentration result in identical values for the three parameters in this relationship, and its validity does not change. Irrespective of this relationship, when first-order kinetics are observed, rate constants and half-lives describe the dissipation profile over the modeled range of substrate concentrations, and provide a valid and well established measure for comparing different decay profiles.

However, to compare the efficiency of enzymatic reactions where different preparations of an enzyme

are used, it is important to consider the concentration and specific activity of different enzyme preparations and their potential effect on the efficiency of the reaction. Under low enzyme concentrations, increasing the concentration or specific activity of an enzyme is predicted to increase the velocity of the reaction and the $V_{\max}$. Thus, estimates of reaction efficiency may differ if the enzyme concentration or specific activity is not held constant across experiments. The relationship between enzyme concentration and reaction velocity is often accounted for by dividing $V_{\max}$ by the enzyme concentration to produce a new parameter (K_{cat}). Enzyme efficiency is then estimated as K_{cat}/K_m .

While this measure of reaction efficiency (K_{cat}/K_m) is valid under conditions of low enzyme concentration, it may not hold true under conditions of high enzyme concentration. Under conditions where the enzyme concentration is saturating, the reaction efficiency becomes insensitive to enzyme concentration, and adjusting estimates of reaction efficiency to account for relatively small differences in enzyme concentration or specific activity is not appropriate. Here, we investigated the effects of varying the pepsin concentration and specific activity in SGF around the level specified in the US Pharmacopeia (0.32%) in an attempt to determine how to best estimate reaction efficiency in this assay and/or

to standardize the concentration of pepsin across experiments.

3.2. Azocoll

Triplicate SGF digestion experiments made with a single preparation of pepsin (90% pure) at a single concentration (0.288%) demonstrated that azocoll decay closely followed first-order kinetics at the substrate concentrations that were assayed (Fig. 1). In addition, digestion efficiencies were consistent even when initial substrate levels were varied (as illustrated by the final absorbance readings). Estimated half-lives from the three separate experiments were 3.09, 3.06, and 3.11 min. Since rate constants and half-lives estimated from first-order reactions are predicted to be independent of substrate concentration, these results further supported the premise that the reaction was first order.

To independently corroborate the measurement of digestion using absorbance readings from chromogenic substrates such as azocoll, a second method was also used to estimate decay for one of the digestion experiments. SDS-PAGE gels of samples produced during the digestion were prepared. Three bands representing different forms of the protein were considered together for this analysis because all digested at similar rates and all forms were putatively labeled with dye (Fig. 2). Azocoll bands were quantified using densitometry, and a half-life (2.93 min) was estimated from the data (Fig. 2). Results from the densitometry method appeared to be consistent with results from the absorbance method, described previously.

The normalization of pepsin preparations was investigated by adjusting concentrations in SGF based on pepsin purity or specific activity (Fig. 3). Estimates of purity and specific activity (hemoglobin method, Anson, 1938) were provided by the supplier (Sigma, St. Louis, MO). Regardless of the normalization procedure, the average half-life of azocoll was estimated to be from 3.01 to 3.44 min with four different pepsin preparations

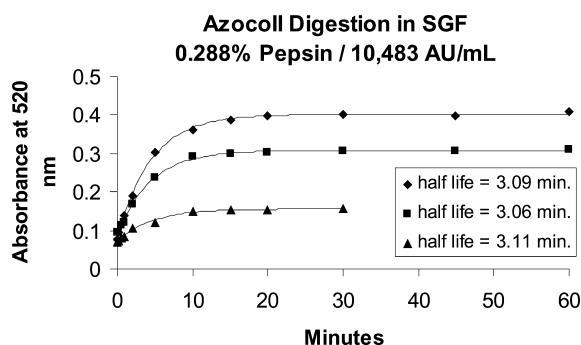


Fig. 1. Digestion of azocoll in 0.288% pepsin (90% purity preparation).

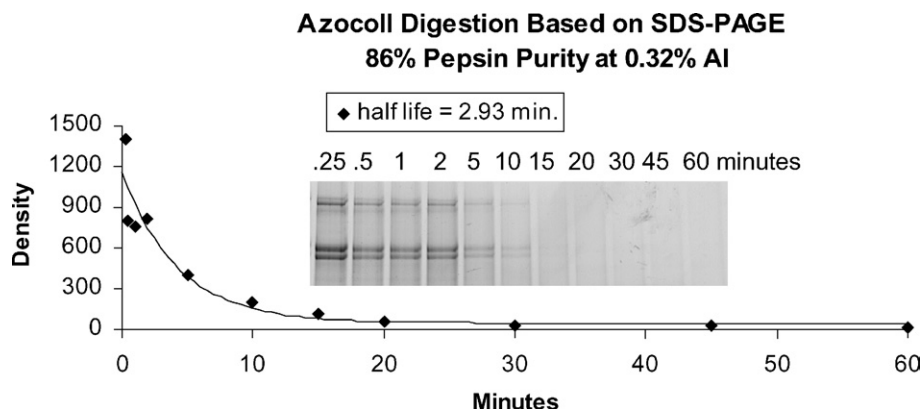


Fig. 2. Azocoll digestion based on SDS-PAGE and densitometry.

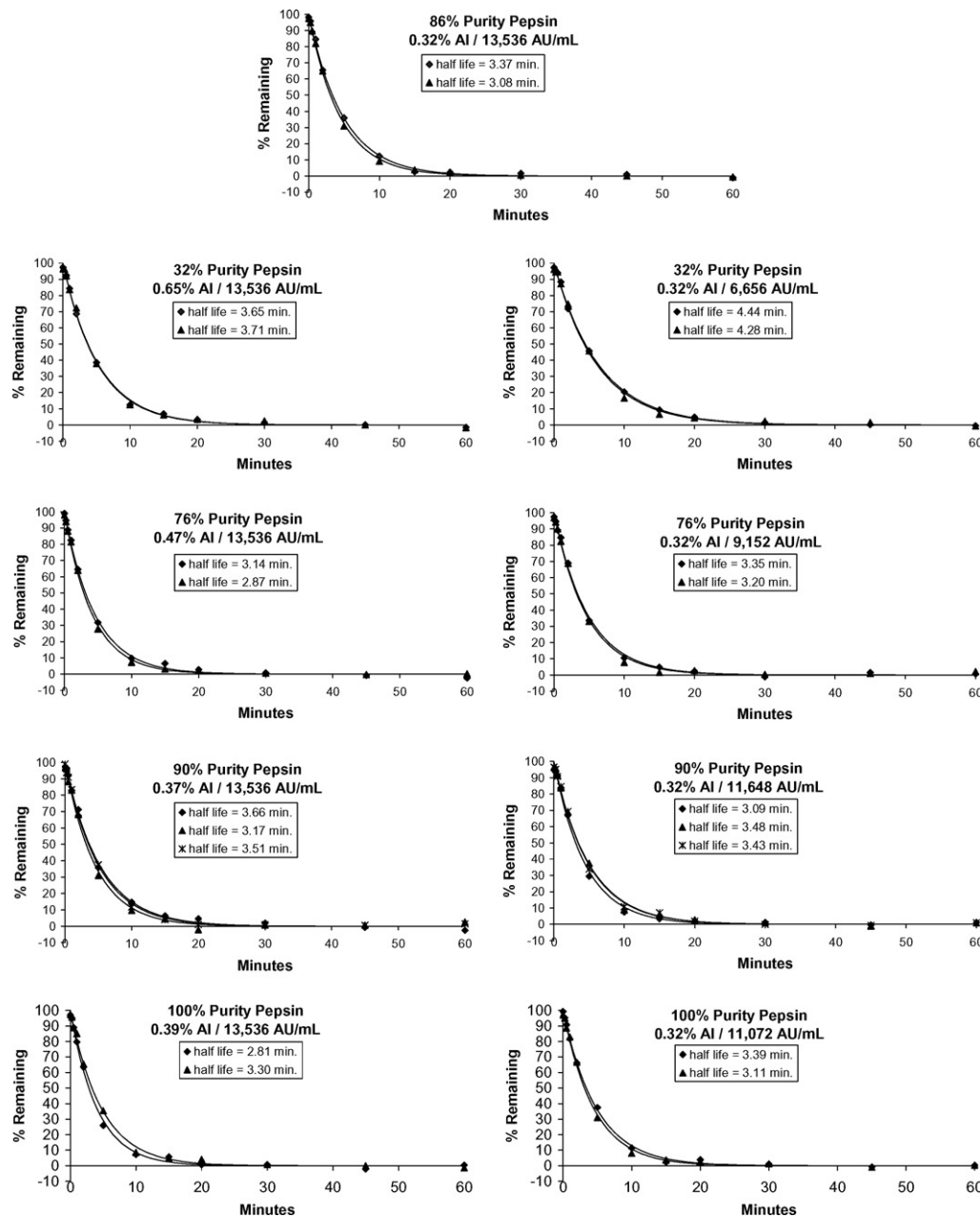


Fig. 3. Digestion of azocoll exposed to different pepsin preparations normalized based on purity or activity to the 86% pure pepsin preparation.

that varied in purity from 76 to 100% (Fig. 4). A 32% pure pepsin preparation was less effective at digesting the azocoll, producing average half-lives of 3.68 and 4.36 min depending on the normalization method (Fig. 4). It appeared that normalizing for pepsin purity or specific activity was not sufficient to compensate for the low purity of the 32% pure pepsin preparation. These results suggest that at the high pepsin concentration (0.32%) specified for simulated gastric fluid (The United States Pharmacopeia 24, 2000), the digestion efficiency for azocoll is relatively insensitive to significant shifts in enzyme concentration.

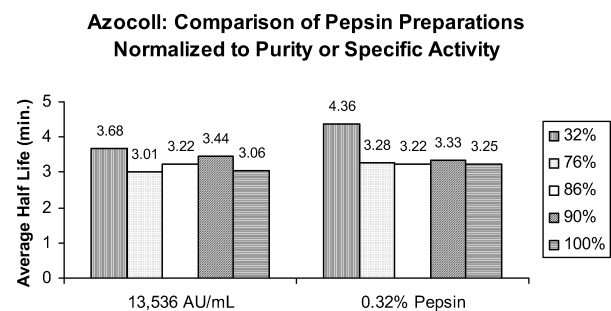


Fig. 4. Average half lives of azocoll exposed to different pepsin preparations normalized based on activity or purity.

3.3. DQ-ovalbumin

Triplicate experiments with a single preparation of pepsin (86% pure) indicated that the digestion of this substrate also behaved according to first-order kinetics (Fig. 5). Two replicate experiments produced half-life estimates of 10.28 and 12.84 min. A third experiment conducted with half the concentration of DQ-ovalbumin (25 $\mu\text{g/mL}$), compared with the first two experiments (50 $\mu\text{g/mL}$), resulted in a half-life estimate of 11.71 min. The relative insensitivity of the digestion effi-

ciency of DQ-ovalbumin in response to a twofold change in substrate concentration provided additional support that the reaction was proceeding according to first-order kinetics.

A similar half-life for non-labeled ovalbumin (12.6 min) was also calculated from the density of SDS-PAGE gel bands as estimated from Fig. 1 in Takagi et al. (2003). These data were rescaled and plotted here along with the best fit of the three-parameter exponential model (Fig. 6). The initial time-zero (neutralized sample) was excluded from the calculations, as was done

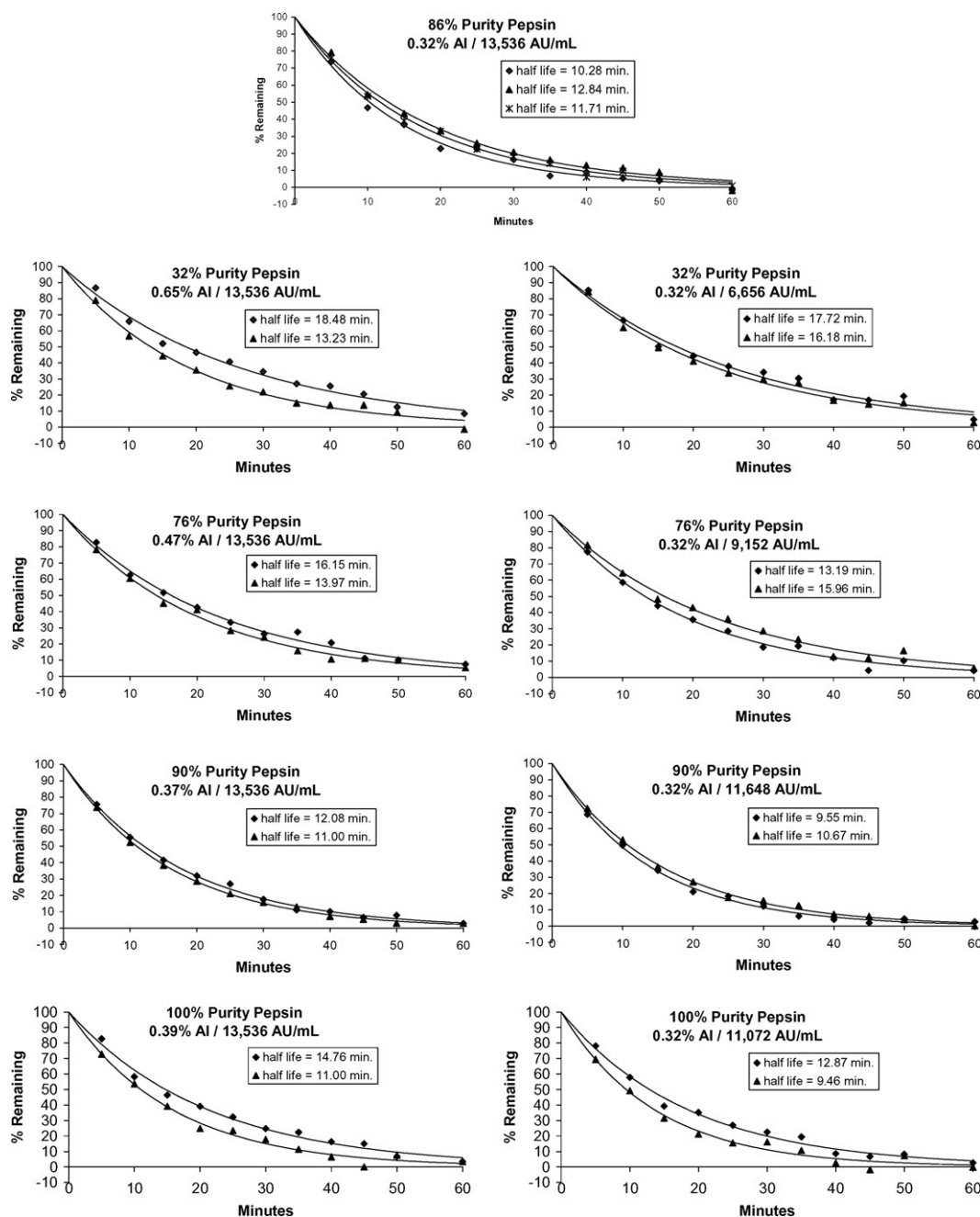


Fig. 5. Digestion of DQ-ovalbumin exposed to different pepsin preparations normalized based on purity or activity to the 86% pure pepsin preparation.

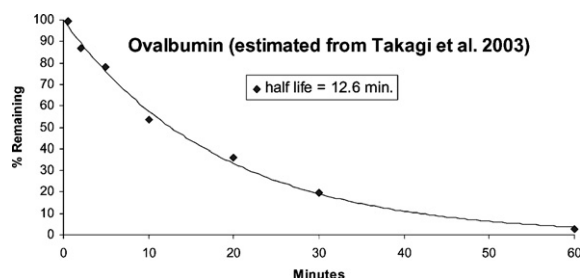


Fig. 6. Re-scaled estimates of ovalbumin digestion from Takagi et al., (2003).

for our analysis, due to a very rapid initial decay. This initial rapid digestion may have been the decay of a pool of denatured or reduced ovalbumin that was generated during purification. Although pepsin and substrate concentrations differed from those used here, and the pH of the SGF was 2.0, the estimated half-life appeared similar to those generated in our studies.

Variability in the digestion efficiency of DQ-ovalbumin among replicate experiments and among pepsin preparations was generally higher than that seen with azocoll (Fig. 5). The lower digestion efficiency with this reaction combined with greater variability in the fluorescence detection method (unpublished) may have contributed to this variability.

As observed for azocoll, the 32% purity preparation of pepsin favored greater persistence of DQ-ovalbumin compared with the higher purity pepsin preparations (Fig. 7). Unlike the azocoll digestion experiments, the 76% preparation also favored greater stability of the DQ-ovalbumin compared to higher purity pepsin preparations. The three highest purity pepsin preparations (86–100%) produced fairly consistent DQ-ovalbumin digestion results when adjusted for either purity (average half-life 10.1–11.6 min) or specific activity (average half-life 11.5–12.9 min). These results further support the observation that the use of high purity pepsin for these experiments promotes greater consistency.

It was also observed that the increasing the pepsin content above the 0.32% specified in The United States Pharmacopeia 24 (2000), may have interfered slightly

with the digestion of DQ-ovalbumin, possibly by causing precipitation of the pepsin or by acting as an alternative substrate. For the four pepsin preparations (32, 76, 90, and 100% purity) where the concentration was adjusted higher than 0.32% to normalize enzyme activity, the digestion efficiency was slightly slower than the matched experiment conducted with the same pepsin preparation at 0.32% in seven of eight experiments.

3.4. Discussion

The SGF assay, when conducted with high purity pepsin preparations adjusted to 0.32% (The United States Pharmacopeia 24, 2000), appears to produce consistent estimates of digestion as long as substrate concentrations are kept low enough to maintain first-order kinetics. Furthermore, under the described conditions, the digestion efficiency for proteins exposed to SGF appear relatively insensitive to significant variation in both pepsin and substrate concentration, and is independent of the analytical method used to estimate decay. Although the pepsin concentration has previously been shown to remain relatively constant for up to 60-min (Herman et al., 2003a), the saturating concentration of pepsin in SGF also mitigates any auto-digestion that might occur with the pepsin. Taken together, these observations suggest that half-lives generated from SGF experiments are useful for making comparisons among different proteins and experiments.

Exploring the relationship between the digestion efficiencies of these chromogenic and fluorogenic substrates, and their reported allergenic potential, is a natural extension of this work. However, binding dye to these proteins may affect digestion. Still, the rapid decay seen here with azocoll (half-life ≈ 3 min) may provide some insight into the relationship between gastric digestion and allergenicity. Collagen allergy, although rare (<2 per million people injected), is well documented, both in relation to food and through injection as a vehicle in vaccines (Pool et al., 2002; Sakaguchi et al., 1996). Collagen is the most prevalent protein in mammals and is the main ingredient in some common foods such as gelatin and some candies. It is also a common constituent of widely used vaccines (e.g., measles–mumps–rubella).

An explanation for why some food allergens may be highly digestible has been presented previously (Yagami, 2002). IgE-mediated allergies result from a two-step process. First an individual is sensitized to the protein, and later exposure to the protein elicits a clinical allergenic response. It is clear that highly digestible proteins may elicit allergenic responses, but it has been suggested that these proteins may not be responsible for the sensitization process (Bannon et al., 2002; Viths, 1997; Yagami, 2002). For example, sensitization by cross-reactive proteins via inhalation (pollen) or dermal exposure (latex)

**DQ-ovalbumin: Comparison of Pepsin Preparations
Normalized to Purity or Specific Activity**

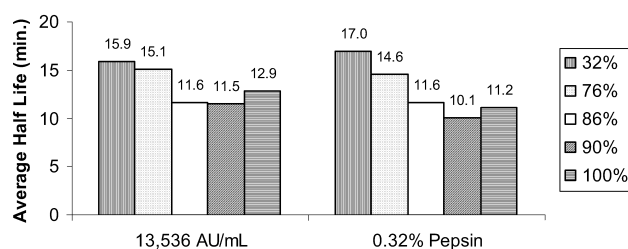


Fig. 7. Average half lives of DQ-ovalbumin exposed to different pepsin preparations normalized based on activity or purity.

has been implicated in food allergy to digestively labile proteins in some fruits and vegetables. Thus, it has been suggested that the relationship between SGF stability and allergenicity may only hold for complete allergens capable of both sensitizing an individual and eliciting an allergic response. An association between patients showing reactions to collagen-containing vaccines and those with collagen food allergy has also been documented (Pool et al., 2002; Sakaguchi et al., 1996). Thus, exposure to collagen via non-oral routes may be responsible for sensitization.

Structural homology between putative sensitizers, to which individuals are exposed by non-oral routes, and food allergens have been documented (Ebner et al., 2001; Midoro-Horiuti et al., 2001; Yagami, 2002). These examples support the weight-of-evidence approach currently used to predict the allergenic potential of novel proteins. This approach includes, among other things, an evaluation of stability in SGF, and structural comparisons with known allergens. The importance of structural-homology data in the evaluation of novel food proteins is highlighted by these examples. We suggest that future comparisons between the digestibility of proteins and their allergenicity consider homology to inhalation, dermal or injection allergens, and that the potential role of food allergens in the sensitization and elicitation process be explored in relation to digestibility. Such consideration may aid in better defining the properties of allergens.

The longer half-life of the DQ-ovalbumin (10–13 min) is consistent with its status as a complete food allergen that can both sensitize and elicit an allergic response (Bannon et al., 2002). However, it should be emphasized that the effects of the bound dye on the digestion of these chromogenic or fluorogenic protein substrates is unknown (However, our results with DQ-ovalbumin are consistent with non-labeled ovalbumin as reported by Takagi et al., 2003). Still the relatively short half-life for the putatively incomplete and rare allergen, collagen, and the relatively longer half-life for the complete allergen, ovalbumin, is consistent with previous reports of a relationship between stability in simulated gastric fluid and the ability of a protein to be a complete allergen (Astwood et al., 1996; Bannon et al., 2002).

4. Conclusions

It is common for enzymatic reactions to proceed according to first-order kinetics when substrate concentrations are low ($\ll K_m$, Schnell and Mendoza, 2004). Here, we confirmed this relationship for two protein substrates (azocoll and DQ-ovalbumin) when exposed to simulated gastric fluid. We further established that estimates of digestion using SDS-PAGE and densitometry are similar to those obtained using dye or fluores-

cence liberation from chromogenic or fluorogenic substrates, although the latter techniques have serious limitations when multiple substrate species or digestion fragments are present. Furthermore, it appears that at the pepsin concentrations used in simulated gastric fluid (0.32%), digestion half-lives may be relatively insensitive to significant deviations in enzyme concentration. The saturating concentration of pepsin in SGF should also mitigate potential kinetic effects that might occur due to competition by stable digestion fragments (if they are present). Subsequent digestion of fragments may also be modeled using kinetic approaches (Atkins, 1986).

This work suggests that the use of first-order rate constants to predict relative digestion efficiencies for novel proteins should typically be appropriate even if protein and enzyme concentrations are both varied among experiments, as long as the pepsin concentration is kept close to the recommended level (0.32%, *The United States Pharmacopeia* 24, 2000), the pepsin preparation is of high activity, and the substrate level ($[S]$) is kept low ($[S] \ll K_m$). First-order rate constants are often good estimates of enzyme efficiency under these conditions (equal to V_{max}/K_m), and are thus meaningful measures for comparing the efficiency of a single enzyme for different substrates (Hansen et al., 2001; Sharp and Rosenberry, 1982; Talanian et al., 1997). Half-lives calculated from first-order rate constants incorporate a measure of the maximum reaction velocity (V_{max}) and the concentration of substrate required to achieve half of that rate (K_m). This index is especially useful under the saturating pepsin concentrations specified for simulated gastric fluid, where the direct relationship between the first-order rate constant and enzyme concentration is often absent (potentially making the use of K_{cat}/K_m as a measure of enzyme efficiency inaccurate). This work further supports a first-order kinetic approach as often being appropriate for comparing the digestibility of proteins in simulated gastric fluid, and confirms that the simulated gastric fluid assay behaves as many other enzymatic reactions (Campaner et al., 1998; Chung et al., 2000; Folco et al., 1997; Fox et al., 2000; Gori et al., 2001; Herman et al., 1998, 2004; Kingston-Smith et al., 2002; Lee et al., 2001; Miller et al., 2002; Potter et al., 2002; Pratt et al., 2002; Schnell and Mendoza, 2004; Sharp and Rosenberry, 1982; Steglich et al., 1999; Talanian et al., 1997; Valentijn et al., 1998; and many others).

As with any approach for tracking the dissipation of a material, sufficient data must be available over a sufficient time frame to evaluate the decay behavior. Thus, half-lives for proteins that digest very quickly may be difficult to obtain due to the need to sample and stop reactions very quickly. Similarly, proteins that digest very slowly may demonstrate very little decay over the chosen duration of a particular experiment. However, for risk-assessment purposes, this is likely to be an insignificant factor since characterizing a particular protein

as very labile or very stable is likely to be sufficient for a weight-of-evidence assessment of allergenic risk. While the examples reported here are limited to simple reactions devoid of digestion fragments, appropriate methods for modeling the appearance and dissipation of such fragments has been described (Atkins, 1986). When digestion fragments are present, methods such as SDS–PAGE followed by densitometry are required to allow profiles for parent protein and fragments to be tracked independently.

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