Comparing two commercial enzymes to estimate in vitro proteolysis of purified or semi-purified proteins

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Running title: Enzymic proteolysis of purified and semi-purified foods
Comparing two commercial enzymes to estimate *in vitro* proteolysis of purified or semi purified proteins

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Summary

Two experiments compared the suitability of two commercial enzymes to estimate *in vitro* proteolysis of different proteins. Experiment 1 compared the proteolytic activity over various incubation times of a microbial enzyme (protease from *Streptomyces griseus*) with a plant enzyme (papain from *Papaya latex*) by using either 1.33 (high, H) or 0.4 (low, L) units (U, amount) of each enzyme per mg crude protein (CP) of purified proteins including bovine (BA) or egg albumin (EA). Experiment 2 compared the activity of 0.66U of each of these enzymes per mg CP of semi-purified proteins including casein, wheat gluten (WG) and maize gluten meal (MG). Each incubation was terminated by adding trichloroacetic acid (TCA) and the TCA soluble supernatant collected to estimate concentration of total amino acids (AA) as the measure of proteolysis of each protein over each time. The data on proteolysis over time were fitted into a non linear model to derive constants for solubility \((a)\) and rate \((c)\) and extents \((a+b)\) of proteolysis of each food by each amount of each enzyme. All data on proteolysis over time and the derived constants were statistically analysed to study the effect of food, enzyme, amount and their interactions. Significant differences were observed between foods, enzymes, enzyme amount (H vs L for experiment 1 only) for the proteolysis at most incubation times in both experiments \((P<0.001)\). The mean proteolysis over all times for BA was 1.6 (SD, 0.52) times greater than EA \((P<0.001)\). While Protease gave about 4 times (SD, 2.1) more proteolysis than Papain \((P<0.001)\), the high amount of enzyme gave only about 2 times (SD, 0.28) greater proteolysis than that of the low amount \((P<0.001)\). On average, the protease was over three times faster \((c)\) than papain \((P<0.001)\) and high amount was 2 times faster than the low amount of enzyme \((P<0.001)\). While both purified foods were similar in solubility \((P>0.05)\), they differed in the rate and extent of proteolysis \((P<0.001)\). BA was degraded about 4 times faster than EA \((P<0.001)\). Amongst semi-pure proteins, casein gave the highest but MG the lowest proteolysis at each incubation \((P<0.001)\). However, the magnitude of proteolysis depended upon enzyme, food and hours of incubation. On average, casein was degraded at a much faster rate than WG or MG by both enzymes. It appeared that the protease and not papain can be used to estimate *in vitro* proteolysis of pure proteins.

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and semi-pure food proteins. However, further studies are needed to standardise the relevant procedures when using protease to estimate proteolysis of ruminant foods.

**Keywords:** In vitro proteolysis, enzyme, protease, papain, food proteins, ruminants

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**Introduction**

Rumen degradation plays a crucial role in the supply of dietary nitrogen (N) to the rumen microbes and undegraded amino acids for direct absorption by the ruminant animals. Therefore all systems of ration formulation for ruminants do separate dietary proteins into quickly- (QDP) and slowly degradable (SDP), un-degradable (UDP) and digestible UDP (DUP) (Verite and Peyraud, 1989; AFRC, 1993; NRC, 1996). *In sacco* method involving rumen incubations of foods in polyester bags has been used for decades to estimate QDP, SDP, UDP, DUP and rate of protein degradation in ruminants (Nocek, 1988; Chaudhry and Webster, 1993; Huntington and Givens, 1995). However, the routine use of the *in sacco* method is constrained by the fact that it requires surgically prepared animals, is laborious, costly and has animal welfare implications. It is therefore essential to develop alternative methods that do not involve surgically modified ruminants to estimate protein degradation of different foods.

An *in vitro* method must be more rapid, reliable, convenient and cost effective than the *in sacco* method and also must be able to replace the need to use animals to study rumen degradation. Many efforts have been made in the past to develop *in vitro* methods by involving either rumen fluid (Broderick, 1978; Nocek, 1988; Luchini et al., 1996) or its enzyme extracts (Mahadevan et al., 1980; Kohn and Allen, 1995) or solvents (Nocek, 1988; Chaudhry and Webster, 2001ab) or commercial enzymes (Poos-Floyd *et al*., 1985; Aufrere *et al*., 1991; Luchini *et al*., 1996; Licitra *et al*., 1999) to estimate *in vitro* degradability of protein foods. While these methods showed variable degrees of success depending upon the type of methods and foods, none of these methods including those requiring enzymes has been adopted for the routine estimation of rumen degradation *in vitro* of a wide range of foods. This may be because an enzyme based method to estimate degradation of a food depends upon not only the source, amount and consistency of an enzyme but also its affinity for a specific type and amount of a food protein. Also, it is possible that an enzyme does not react equally and optimally with different food proteins when non protein components are also present in those foods and thus limits its use in a method for adoption to estimate *in vitro* degradation of various foods (Luchini et al., 1996).

It would be appropriate to test the potential of an enzyme based method by using purified and semi-purified food proteins as substrates. Since the non protein components either do not exist or exist only in minimal amounts in these proteins.
substrates, it is almost unlikely to expect interference with the efficacy of an enzyme to estimate in vitro proteolysis of such food proteins. Therefore, the first objective of this study was to test the suitability of two amounts of two commercial enzymes to estimate in vitro proteolysis of purified proteins over various times. The second objective was to select the most suitable type and amount of an enzyme and test its efficacy and reproducibility to estimate in vitro proteolysis of semi purified proteins over different times. The ultimate objective was to establish optimal treatment conditions for an enzyme based method by involving semi purified food proteins before evaluating this method to estimate in vitro degradation of most commonly used ruminant foods.

Materials and methods

Phosphate Buffer (pH 6.8) and chemical solutions

Two stock solutions each of 0.1 molar (M) strength were prepared by mixing separately 13.6g of KH$_2$PO$_4$ and 4g of NaOH in each litre (L) of distilled water. About 500ml of KH$_2$PO$_4$ and 224ml of NaOH stock solutions were then thoroughly mixed with 274ml of distilled water to prepare each L of phosphate buffer of pH 6.8 (buffer) (Dawson et al, 1987). TCA solution (50%) was prepared by dissolving 500g TCA in each litre of distilled water.

Enzyme solutions

Freeze dried samples of a protease from *Streptomyces griceus* (Protease, P5147) and papain from *Papaya latex* (Papain or Papainase P3375) were purchased from Sigma-Aldrich Ltd in Dorset, UK to prepare fresh enzyme solutions or suspensions. The enzymes contained about 75-80% CP and were expected to have activities of about 4 and 3.5 units /mg solid of protease and papain respectively. Here, each unit of enzyme is defined by Sigma as follows: For Papain, one unit will hydrolyse 1.0 µmole of BAEE (Nα benzyle arginine ethyl ester) per minute at pH 6.2 at 25°C and for Protease one unit will hydrolyse casein to produce colour equivalent to 1 µmole or 181µg of tyrosine per minute at pH 7.5 at 37°C. About 300mg of each enzyme was dissolved in 100ml of the buffer to achieve the enzyme concentration of 12U ml$^{-1}$. This solution was freshly diluted with the same buffer to achieve 4U ml$^{-1}$ enzyme solution when needed to incubate purified and semi purified protein foods in the following sections.

Purified proteins and their suspensions

Freeze dried samples of purified food proteins including bovine albumin (BA, A8022), egg albumin (EA, A5503), casein (CA, C7078) and wheat gluten (WG, G5004) were bought from Sigma-Aldrich UK Ltd whereas maize gluten meal (MG) was obtained from Dalgety Agriculture Ltd, UK. The crude protein (CP) contents (g/kg) of these foods were 960, 980, 900, 800 and 750 for BA, EA, CA, WG and MG respectively. Appropriate amounts of these foods to provide
around 15mg CP were used as suspensions in the buffer before their *in vitro* incubation with different enzyme solutions as described in the following sections.

**Experimental design**

*Experiment 1* compared the proteolytic activity of the microbial Protease with the plant Papain by using different amounts in units (U) of enzyme to food-CP ratios. The study was conducted according to a 2x2x2x7 factorial arrangement, in duplicate, by involving 2 albumins (BA and EA), 2 enzymes (Protease and Papain) each at 2 amounts (high= 1.33U and low=0.4U) and 7 incubation times (0, 0.5, 1, 2, 4, 6 and 18h). *Experiment 2* tested the proteolytic activity of the enzymes used in Experiment 1 but at a fixed amount of 0.66U mg⁻¹ food-CP. This study was conducted according to a 3x2x7 factorial arrangement, in duplicate, by including 3 foods (CA, WG and MG), 2 enzymes (Protease and Papain) and 7 incubation times (0, 1, 2, 4, 6, 20 and 30h).

**Experimental Procedures**

In vitro Enzyme incubations

During each of the above experiments each purified or semi purified protein food containing about 15mg CP was weighed separately into a 20ml test tube containing 5ml of the buffer pre-warmed at 39°C. After re-hydration of each food in the buffer for 1 h at 39°C, different volumes of enzyme solutions were added to achieve the respective enzyme amounts. For example to achieve 1.33, 0.66 and 0.4U of enzyme mg⁻¹ food CP, about 5, 2.5 and 1.5 ml respectively of relevant enzyme solution was added to relevant tubes containing a particular food. The volume in each tube was adjusted to 10ml with additional buffer. The test tubes were screw capped, shaken and incubated at 39°C for the preplanned times. No preservatives as antimicrobials were added to these tubes. However, duplicate sets of blanks and controls for each enzyme and protein food were run simultaneously to correct for pre-existing soluble components and microbial growth which was unlikely to occur under these incubations. After each incubation time, the reaction was stopped by adding 1 ml of 50% TCA, cooled in ice and centrifuged at 2500 g for 10 minutes to obtain the supernatants.

**Simultaneous determination of ammonia and amino acids**

The supernatants from each treatment combination and their respective controls and blanks after each incubation time were analysed simultaneously for mM of ammonia (AM) and Leucine (LU) as the representative of total amino acids (AA) by using respectively the phenol -nitroprusside-hypochlorite (Fawcett and Scott 1960) and ninhydrin methods on an automated system as described by Broderick and Kang (1980) after some modifications. This system was equipped with Technicon Autoanalyzer dual system of manifolds involving two colorimeters each of which was linked to a
separate chart recorder. Leucine has been reported to give over 95% theoretical reproducible colour yield with ninhydrin
(Moore and Stein 1954) and so was considered appropriate representative of total AA in this study and elsewhere
(Broderick and Kang 1980). Working standards (0 to 10 mM) of both AM and LU were prepared by diluting relevant
stock solutions (100 mM) containing 0.6607 g (NH₄)₂SO₄ and 1.1312 g methionine-free L-leucine respectively in each
100 ml of 0.1 Normal HCl. These standards were also run with each batch of the samples. Each litre of ninhydrin reagent
that was used to estimate mM of LU contained 6.67 g ninhydrin crystals (Sigma Ltd UK), 500 ml methyl cellosolve (low
peroxide), 28.33 ml glacial acetic acid, 100 ml of sodium acetate buffer (4M with pH 5.5, Dawson et al 1984) and about
371.67 ml of distilled water. The presence as mM of AM and total AA in each supernatant were then estimated by
comparing the individual peaks with the respective peaks for ammonia and leucine standards.

Calculations and statistical analysis

Since there were no changes in AM concentration over time for both experiments, only the data on LU to represent total
AA were used as measures of enzymic proteolysis of foods. The enzyme proteolysis of each protein food by each
amount of each enzyme for each incubation time was calculated by using the corresponding data on the release of mM
of AA in the following equation:

\[
Enzymic \ proteolysis = \frac{mM \ of \ AA \times 1.30 \times 0.014 \times 6.25}{1.364} \times 1000 \tag{1}
\]

where 1.3 = mM of N per mM of protein AA as on average one mole of protein AA contains 1.3 moles of N;
0.014 = molecular weight in g of 1 mM N; 6.25 = factor to convert N into CP and 1.364 = g Food CP in one litre
solution since 15 mg or 0.015 g CP was used in 11 ml solution = (0.015/11) X 1000

The data of enzyme proteolysis from each experiment were fitted according to a model \[ p = a + b (1-e^{-ct}) \] by using Gauss-
Newton method of non-linear regression in SAS. This model did not include the lag phase which could have changed
some aspects of this study. However, the model was able to derive constants for immediate solubility (a) and rate (c) and
extent (a+b) of proteolysis for each treatment combination within each experiment and so its use was appropriate to
match the objectives of this study. The values of potential proteolysis (P₀.₀₆) were also determined by using the relevant
a, b and c values in equation \[ P = a + \{b \times c \}/(c+k) \] where k was assumed as the turnover rate of 0.06/hr. These constants
together with P₀.₀₆ values and the data on proteolysis at each incubation time were then statistically analysed by using
GLM procedures in SAS. The model compared the main effect of Food protein type (F), Enzyme type (E), enzyme
amount or volume (V) and their interactions on the proteolysis at each incubation time and the derived constants. Since
there was only one level of enzymes used in experiment 2, only the main effect of F and E and their interaction were included in the model for data from this experiment. The significance of an effect was declared if $P<0.05$.

**Results**

**Experiment 1**

All main effects of food (BA v EA), enzyme type (Protease v Papain), enzyme amount (H v L) and their 2 & 3 way interactions were significant for the proteolysis at most times of incubation ($P<0.001$). Therefore, the mean proteolysis data for each treatment combination (FxExV interaction) and not for the main factor effects are presented as Figure 1 (BA) and Figure 2 (EA). The relevant standard errors to compare data in Figures 1 & 2 for 0, 0.5, 1, 2, 4, 6 and 18 hours of incubations were 1, 3.5, 3.52, 0.89, 1.3, 3.4, and 4.8 respectively. Bovine albumin (BA) was degraded to a larger extent than EA by both levels of Protease at all but 0 hours of incubation whereas EA was degraded more than BA by both ratios of Papain at most hours of incubation. Each ratio of Protease gave higher proteolysis for each food than the corresponding amounts of Papain at all but 0 hours of incubation. Here the proteolysis was marginally (se 1.0, $P>0.05$) higher for EA at low amount of Papain than the corresponding proteolysis of both BA and EA at the low amount of Protease. Clearly, the proteolysis increased with time for both foods in response to each amount of each enzyme. Although, the proteolysis of EA by Protease in Figure 2 did not reach a plateau even at 18h of incubation, the proteolysis by Protease (Figures 1 and 2) was much higher than its Papain counterparts, particularly from 1 or 2 hours of incubation and beyond. On average, BA was degraded 1.6 (SD, 0.52) times more than EA ($P<0.001$). While Protease gave over 4 (SD, 2.1) times higher proteolysis than Papain ($P<0.001$), the proteolysis at the high amount of enzyme was only about 2 times (SD, 0.28) higher than that of the low amount ($P<0.001$).

Table 1 presents least square means of constants being derived by fitting the data on in vitro proteolysis in Figures 1 and 2 as described earlier.

Bovine albumin (BA) was 37% more soluble (a) than EA at the high amount of Protease whereas EA was 17% more soluble than BA at the low amount of Protease and from 16 to 25% more soluble at respectively low and high amounts of Papain ($P<0.001$, Table 1). However, on average, the differences between solubility were not significant ($P>0.05$) for either foods or enzymes but significant for amounts ($P<0.001$) where the high amount gave two times more solubility than the low amount ($P<0.001$). Despite an ExV interaction, it appeared in Table 1 that the extent (a+b) of proteolysis by Protease was much greater than Papain at each amount. While the mean a+b value for Protease was over 6 times
more than that for Papain (P<0.001), it was only 1.3 times (26%) more for high amount than the low amount (P<0.001) and only 1.1 times (6%) more for EA that of BA (P<0.01). The rate of proteolysis (c) for Protease was far greater than its Papain counterparts but there were a few exceptions where Papain was either similar or slightly better than Protease depending upon the type of food and enzyme amount (P<0.001, Table 1). On average, the protease achieved over three times greater rate of proteolysis than that of papain (P<0.001) whereas high amount gave 2 times faster proteolysis than the low amount of enzyme (P<0.001). While both foods were similar in solubility (P>0.05), they differed in the rate and extent of proteolysis (P<0.001). In fact, BA was degraded about 4 times faster than EA (P<0.001). Although the values of potential proteolysis or degradability (P_{0.06}) followed the same pattern to those of a+b values, these were lower than those of a+b values for each treatment combination.

Table 1 about here

Experiment 2

Figure 3 about here

The proteolysis for Protease was significantly greater than Papain at most times but was also showing interactions between Food and Enzyme types at all (P<0.001) but 20 (P>0.05) hours of incubation and so the proteolysis for each treatment combination at each hour of incubation are presented in Figure 3. On average, the proteolysis by Protease was about 6 to 12 times higher than that of Papain over various times (P<0.001). Casein gave the highest but Maize gluten the lowest proteolysis at each hour of incubation (P<0.001). However, the magnitude of the difference between these foods for proteolysis was dependant upon the hours of incubation.

Table 2 about here

The main effects of Food and Enzyme and their F*E interaction were significant for all constants and P_{0.06} (P<0.01) (Table 2). On average, Protease gave 3.5 times more solubility (a) than Papain (P<0.001). Casein was about 4 and 10 times more soluble than maize gluten (P<0.001) and wheat gluten respectively (P<0.001) whereas maize gluten was about 3 times more soluble than wheat gluten (P <0.01). Protease gave highest a+b values for casein and lowest for maize gluten whereas Papain gave the highest a+b for maize gluten and the lowest for wheat gluten showing a F*E interaction (P<0.01). However, on average Protease gave over 5 times higher a+b than that of Papain (P<0.001). In addition, a+b for casein was about 20% (P<0.01) and 33% (P<0.001) more than that of maize gluten and wheat gluten respectively whereas a+b for maize gluten was only 11% higher than that of wheat gluten (P>0.05). The rate of proteolysis (c) by Protease for casein was similar to that for wheat gluten (P>0.05) but was significantly higher than that
for maize gluten (P<0.001) but by Papain for casein was significantly higher than those for maize gluten and wheat gluten (P<0.001) and thus showing F*E interaction (P<0.01). However, on average, the rate of proteolysis for Protease (c=0.295) was about 2.1 times higher than that (c=0.138) of Papain (P<0.001). The rate of proteolysis was highest for casein (0.374) followed by wheat gluten (0.217) and lowest for maize gluten (0.059). Although the values of potential proteolysis or degradability (F_f) followed the same pattern as those of a+b values, these were lower than those of a+b values for each relevant treatment combination.

Discussion

The study demonstrates that different enzymes are not equally reliable for estimating proteolysis of foods. Clearly, a single enzyme, particularly protease, based method can be used to differentiate between various substrates within pure or semi pure proteins on the basis of their in vitro proteolysis or degradation. It also appeared that TCA soluble AACP in supernatants instead of residual N could be used to monitor degradation of such foods over time. It might have been helpful to use an alternative model to conform to all the data or increase the duration of incubation for some combinations in Figure 2 to achieve a plateau as shown for the data in Figures 1 and 3 in the exponential model of this study. However, this alternative approach may not have changed the overall conclusions of this study. In fact, the exponential model under report was able to differentiate between the efficacy of two different types and amounts of enzymes in determining the in vitro degradation of various substrates. However, the extent of variation in the rate and extent of degradation was dependant upon the type and amount of an enzyme, food substrate and their interactions. Such differences between the proteolytic activities of different enzymes are not uncommon. Previous studies have shown similar variations in rates and extents of proteolysis for different foods when enzymes were used either in isolation (Poos-Floyd et al., 1985; Licitra et al., 1999) or in combination (Luchini et al., 1996). The variation between different foods for proteolytic rates may be attributed to the variation in solubility, type, size and structure of proteins in these foods (Mahadevan et al., 1980; Aufrere et al., 1991; Chaudhry and Webster 2001a, b).

The low ‘a’ values (g/kg) for purified foods including EA and BA (23 to 62, Table 1) and for semi-purified foods involving casein, WG and MG (2 to 32, Table 2) suggested that these foods did not contain high amounts of TCA soluble free AACP or N. In fact, the amounts of soluble component ‘a’ in semi-pure foods were even lower than their pure counterparts. These observations support the assumption that the foods of this study represented two separate groups which were distinctly different and therefore showed variation in soluble components. However, the presence of even small amounts of soluble fraction representing free AA in the absence of enzymic action was surprising and
unexpected. Clearly, the marked differences in degradation over time (Figures 1 and 2), extent of degradation (a+b) and $P_{0.06}$ between pure (Table 1) and semi-pure (Table 2) foods were the direct results of variable proteolytic activities of different amounts and types of these two enzymes. Similar differences in the extent of protein degradation of different foods by the same enzyme or the same food by different enzymes have been reported in various situations (Mahadevan et al., 1980; Kohn and Allen, 1995; Luchini et al., 1996; Licitra et al., 1999). It is postulated that the protein degradation was directly related to the solubility of various foods (Greeberg and Shipe, 1979; Marais and Evenwell, 1983; Chaudhry and Webster, 2001a) and their interactions with the level and type of different enzymes (Aufrere et al., 1991).

The rates (c) of degradation of casein for each enzyme were faster than those of WG and MG. This is in agreement with Mahadevan et al., (1980) who observed the greatest per hour release of amino acids from casein on incubation with *Bacteroid amylophilus* protease compared with many other substrates. The higher *in vitro* proteolysis by protease compared with its papain counterpart for each substrate suggests that protease from *S. griseus* was more suited to degrade these foods. However, Luchini et al., (1996) observed even greater degradation rates for different foods by strained rumen fluid (SRF) compared with the protease from *S. griseus*. This greater proteolytic activity of SRF was expected because SRF contains mixed rumen microbes which would work better in consortium because of their complementary proteolytic actions than that of a single enzyme. In contrast the enzyme based degradation rates (per hour) of 0.36-0.76 for Bovine albumin of this study (Table 1) were much greater than 0.086-0.139 being reported by Broderick and Clayton (1992) for SRF. Similarly, the enzymic degradation rate for MG of 0.078 (Table 2) was much greater than 0.03 being reported for the *in sacco* method by AFRC (1993). Moreover, the enzymic degradation rate of 0.416 for casein (Table 2) was either comparable to 0.395 (Broderick and Clayton, 1992) or greater than 0.24 (Luchini et al., 1996) being reported for different SRF based in vitro methods. Such differences in proteolysis by different methods of similar foods could be attributed to the variation in methodology involving either pure enzymes (Aufrere et al., 1991; Luchini et al., 1996; Licitra et al., 1999) or various SRF and their extracts (Broderick and Clayton 1992; Kohn and Allen, 1995; Luchini et al., 1996; Hristov et al., 2002). The variable rates of degradation of MG may be because different samples contained different amounts of zein which due to its high disulphide bonds showed variable resistant to degradation by enzyme or SRF.

*[Figure 4 and Figure 5 about here]*
Although papain was able to degrade various protein foods over time (Figures 1-3) and on average showed a similar solubility (Figure 4), it was unable to match the proteolytic activity (a+b) of the protease for either each food for each time (Figures 1-2) or their overall average (Figure 4). While, the rates and extents of degradation were dependant upon the amount of the protease and the type of a food substrate, the change in the extent of proteolysis was not proportionate to the change in the amount of the protease being used in this study (Tables 1 & 2). In contrast, when the data on rate of proteolysis was averaged for each amount of each enzyme over all foods and times of both experiments, there was clearly a curvilinear ($R^2=0.99$) increase in the mean rate of proteolysis with the increase in the amount of protease from 0.4 to 0.668 to 1.332 Units /mg CP (Figure 5). However, the papain was unable to show a similar trend ($R^2 = 0.38$).

Ideally the amount of protease to be used in the *in vitro* methods should be closer to an *in vivo* system. However, it is difficult to use a precise amount of such enzymes because an *in vivo* system is dynamic and so is bound to change with animal, diet and various metabolic activities. The amounts of the protease used in this study ranged from 0.4 to 1.332 U /mg food CP which were equivalent to 0.55 to 1.8U /ml and so were much closer to those of 0.6 to 2U /ml that were used previously of a different batch of the same protease (Licitra et al., 1999). However, the methodology employed in this study to estimate proteolysis was substantially different from many of the previously reported methods (Aufrere et al., 1991; Cone et al., 1996; Licitra et al., 1999). Most methods employed insoluble N in residues following incubation of foods with enzymes whereas in this study the TCA soluble N was used instead to estimate protein degradation by measuring leucine equivalent AA as described by Broderick and Kang (1980).

Figure 6 present mean potential degradability ($P_{0.06}$) for each amount of each enzyme.

Clearly $P_{0.06}$ values increased with increasing amount of each enzymes but the extent of increase was not proportionate to the increase in the amount of enzyme and hence showing a curvilinear relationship with the amount of protease ($R^2=0.99$) and Papain ($R^2 =0.9$). Overall average of $P_{0.06}$ for Protease was over 6 times more than Papain confirming the fact that only Protease and not Papain was reliable for estimating proteolysis of foods. The only $P_{0.06}$ values involving either in sacco or in vitro methods using enzymes or SRF being reported in literature were those for maize gluten meal amongst different protein foods that were tested in this study. On comparison with these literature values, it appeared that $P_{0.06}$ (g/kg) of maize gluten meal at the estimated turnover rate of 0.06 of this study (200) was either within the range of 70-390 reported by Licitra et al., (1999) or greater than 80-120 (Licitra et al., 1999) for the similar protease
involving different in vitro methods but was much lower than 270 reported by AFRC (1993) and 320-450 reported by Licitra et al (1999) for in sacco method.

In summary, this study was able to show that the amounts of protease used in this study were appropriate to differentiate between the proteolysis of various foods over time. However, it would be essential to test the reproducibility of this method by involving a wide range of protein foods to estimate their degradability for ruminants in the future studies. Also, it would be helpful if longer hours of incubation are used to achieve a plateau if the same exponential model is employed in future studies of this kind.

Conclusion

Clearly, Papain was unable to show as much response as was shown by Protease and therefore seemed not to be suitable to obtain reliable estimates of degradability of various foods. In contrast Protease, despite variation between foods for degradation, showed a good potential in estimating degradation of pure and semi pure protein foods. However, it would be essential to standardise the treatment conditions to test the reproducibility of Protease by using a wide range of foods before recommending its routine use to estimate degradation of different ruminant foods.

Acknowledgments

ASC thanks Dalgety Agriculture Ltd. for funding, Professor A.J.F. Webster for initial discussions and Mike Kitchenside and Liz Glen for their assistance in laboratory analysis during this work at the University of Bristol, UK.

References


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Figure 1.

Caption for Figure 1

Experiment 1. *In vitro* proteolysis of Bovine albumin over various hours of incubation by high (H= 1.334 units or U) or low (L, 0.4 U) amount of protease or papain per mg albumin-CP.
Figure 2.

Caption for Figure 2.

Experiment 1. In vitro proteolysis of Egg albumin over various hours of incubation by high (H= 1.334 units or U) or low (L, 0.4 U) amount of protease or papain per mg albumin-CP.
Table 1 (Experiment 1).

Least Square (LS) means (g AACP/kg CP) for different constants derived by fitting data on *in vitro* proteolysis from Figures 1 & 2 exponentially together with standard errors (SE) or significance for the main effects of Food (F), Enzyme type (E) and ratio (V) and their 2 and 3 way interactions.

<table>
<thead>
<tr>
<th>Food (F)</th>
<th>Bovine Albumin</th>
<th>Egg Albumin</th>
<th>SE</th>
<th>Significance</th>
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<td>Level (V)</td>
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| Constants |
|__________|
| __________ | ______________ | __________ | ____ | ________________________________ |
| a | 62 | 23 | 48 | 25 | 45 | 27 | 60 | 29 | 1.24 | NS | NS | *** | *** | ** | NS | *** |
| b | 406 | 363 | 35 | 20 | 432 | 339 | 39 | 24 | 4.12 | ** | *** | *** | * | NS | *** | NS |
| a+b | 468 | 386 | 83 | 44 | 477 | 410 | 99 | 53 | 4.63 | ** | *** | *** | NS | NS | ** | NS |
| c | 0.76 | 0.36 | 0.16 | 0.11 | 0.17 | 0.06 | 0.083 | 0.049 | 0.017 | *** | *** | *** | NS | NS | ** | *** | ** |
| P<0.06 | 438 | 334 | 73 | 37 | 366 | 221 | 82 | 39 | 3.3 | *** | *** | *** | *** | *** | *** | ** |

Here a= intercept representing solubility or proteolysis; a+b= asymptote or potential proteolysis; c= proteolytic rate of b of a+b; P<0.06= Predicted degradability at turnover rate of 0.06 per hour; H and L are High (1.334) and Low (0.4) amounts (units, U) of enzyme per mg Food CP; NS and *, ** or *** are non-significant and significant at P<0.05, P<0.01 and P<0.001 respectively. SE’s for main effects were 0.62, 2.1, 2.3, 0.009 and 1.67 for a, b, a+b, c and P<0.06 respectively.
Figure 3 (Experiment 2)

*In vitro* proteolysis (mean±SE) of casein (Ca), wheat gluten (Wg) and maize gluten meal (Mg) by protease (Pr) and papain (Pa) at various hours of incubation. The relevant SE’s (*P<0.001 at most times*) to compare individual means on this graph were 2.7, 4.4, 10.3, 6.4, 7.3, 16.0 and 9.1 respectively for 0, 1, 2, 4, 6, 20 and 30 hours of incubations. Here the original data points are represented by symbols together with error bars whereas the fitted \( p = a + b \left( 1 - \exp^{-ct} \right) \) data is presented by solid or broken lines.
Table 2. Experiment 2.

Fitted constants for in vitro Proteolysis (g AA CP /kg Food CP) of foods as indicated by ‘A’ (immediate proteolysis or solubility), ‘C’ (rate) and ‘A+B’ (extent)

<table>
<thead>
<tr>
<th>Food  (F)</th>
<th>Casein</th>
<th>Wheat gluten</th>
<th>Maize gluten</th>
<th>SE</th>
<th>Main effects &amp; Interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme (E)</td>
<td>Protease Papain</td>
<td>Protease Papain</td>
<td>Protease Papain</td>
<td>F</td>
<td>E</td>
</tr>
<tr>
<td>a (Solubility)</td>
<td>32</td>
<td>8</td>
<td>1.8</td>
<td>2.4</td>
<td>9.8</td>
</tr>
<tr>
<td>b</td>
<td>434</td>
<td>61</td>
<td>355</td>
<td>43.4</td>
<td>338</td>
</tr>
<tr>
<td>a+b (Extent)</td>
<td>466</td>
<td>69</td>
<td>357</td>
<td>46</td>
<td>348</td>
</tr>
<tr>
<td>c (rate per hour)</td>
<td>0.416</td>
<td>0.332</td>
<td>0.389</td>
<td>0.044</td>
<td>0.078</td>
</tr>
<tr>
<td>$P_{0.06}$</td>
<td>411</td>
<td>60</td>
<td>308</td>
<td>21</td>
<td>200</td>
</tr>
</tbody>
</table>

Here only a fixed amount of 0.66U of enzyme per mg Food-CP was used for each treatment combination; ns and *, ** or *** are non-significant and significant at P<0.05, P<0.01 and P<0.001 respectively. SE is the standard error.
Figure 4.

Solubility (A) and extent of proteolysis (A+B) for protease and papain when averaged (mean ± SD) over all foods and ratios that were used in experiment 1 and experiment 2.

![Solubility (a) and proteolysis (a+b) graph](image-url)
Caption for Figure 5

Rate (c) of proteolysis of each enzyme at each amount averaged over all foods of experiment 1 & 2.
‘Caption for Figure 6’

Figure 6

Mean $P_{0.06}$ values (g/kg) for each amount of Protease or Papain when averaged over all foods of experiment 1 and experiment 2.