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Effect of quantifying peptide release on ruminal protein degradation determined using the inhibitor in vitro system[1]

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ABSTRACT

The aim of this work was to compare use of an *o*-phthaldialdehyde (OPA) colorimetric assay (OPA-C), which responds to both free AA and peptides, with an OPA fluorimetric assay (OPA-F), which is insensitive to peptides, to quantify rates of ruminal protein degradation in the inhibitor in vitro system using Michaelis-Menten saturation kinetics. Four protein concentrates (expeller-extracted soybean meal, ESBM; 2 solvent-extracted soybean meals, SSBM1 and SSBM2; and casein) were incubated in a ruminal in vitro system treated with hydrazine and chloramphenicol to inhibit microbial uptake of protein degradation products. Proteins were weighed to give a range of N concentrations (from 0.15 to 3 mg of N/mL of inoculum) and incubated with 10 mL of ruminal inoculum and 5 mL of buffer; fermentations were stopped after 2 h by adding trichloroacetic acid (TCA). Proteins were analyzed for buffer-soluble N and buffer extracts were treated with TCA to determine N degraded at t = 0 (FD0). The TCA supernatants were analyzed for ammonia (phenol-hypochlorite assay), total AA (TAA; OPA-F), and TAA plus oligopeptides (OPA-C) by flow injection analysis. Velocity of protein degradation was computed from extent of release of 1) ammonia plus free TAA or 2) ammonia plus free TAA and peptides. Rate of degradation (k_d) was quantified using nonlinear regression of the integrated Michaelis-Menten equation. The parameters K_m (Michaelis constant) and k_d ($Vmax/K_m$), where Vmax = maximum velocity, were estimated directly; k_d values were adjusted (Ak_d) for the fraction FD0 using the equation $Ak_d = k_d - FD0/2$. The OPA-C assay yielded faster degradation rates due to the contribution of peptides to the fraction degraded (overall mean = 0.280/h by OPA-C and 0.219/h by OPA-F). Degradation rates for SSBM samples (0.231/h and 0.181/h) and ESBM (0.086/h) obtained by the OPA-C assay were more rapid than rates reported by the National Research Council (NRC). Both assays indicated that the 2 SSBM differed in rumen-undegradable protein (RUP) content; the more slowly degraded SSBM had RUP content (35% by OPA-C) similar to that reported by the NRC. The RUP content of ESBM (42% by OPA-C) was lower than the NRC value. Preliminary studies with 4 additional protein concentrates confirmed that accounting for peptide formation increased degradation rate; however, a trend for an interaction between assay and protein source suggested that peptide release made a smaller contribution to rate for more slowly degraded proteins. The OPA-C assay is a simple and reliable method to quantify formation of small peptides.
Key words: peptide, ruminal protein degradability, inhibitor in vitro system

INTRODUCTION

The in situ method is widely used to determine rate of ruminal protein degradation (Ørskov and McDonald, 1979); however, several factors (such as porosity of bag material, ratio of sample weight-to-bag surface area, particle size of sample and bacterial attachment to feed residues) affect estimates of protein degradation. The method uses a single passage rate, 3 N fractions with the soluble fraction assumed to be totally degraded, and a unique degradation rate applied to the potentially degradable fraction. Gierus et al. (2005) reported similar degradation rates for fine particles escaping through the nylon mesh and the residue retained on the mesh. Further, Aufrère et al. (2002) showed that between 7 and 13% of the nonammonia N disappearing from nylon bags escaped degradation in the rumen. As a consequence, the in situ method can result in inaccurate estimates of RUP and no relationship between ruminal escape N from in situ measurements and in vivo data has been shown (Gosselink et al., 2004).

Broderick (1987) developed an inhibitor in vitro (**IIV**) method in which substrate-limiting amounts of protein were incubated with ruminal inocula containing

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metabolic inhibitors to obtain quantitative recovery of protein breakdown products. This approach has several limitations: 1) accumulation of ammonia, AA and small peptides may make the system subject to end product inhibition; 2) degradation rates determined for feeds containing high levels of ammonia and free AA are less accurate because breakdown of more slowly degraded residual protein must be quantified from appearance of additional ammonia and AA in the presence of high backgrounds; and 3) estimation of degradation rates from the gentle slopes obtained with slowly degraded proteins may be less accurate. An IIV approach based on Michaelis-Menten enzymatic kinetics was developed to circumvent the disadvantages of the substrate-limiting approach (Broderick and Clayton, 1992). However, no accounting was made of peptide formation within the IIV method. Broderick and Wallace (1988) quantified ruminal peptide formation in sheep supplemented with different N sources and concluded that rapidly degraded proteins, such as casein, yielded substantial amounts of peptides. Volden et al. (2002) provided evidence that small peptides, rather than free AA, were the main end product of ruminal proteolysis and an average of 3.7% of total nonammonia N flowed from the rumen as peptide N in dairy cows fed grass silage and concentrate diets (Choi et al., 2003). Hence, the peptides produced during ruminal protein degradation should be determined.

Ruminal peptides are usually quantified from AA released before and after acid hydrolysis of deproteinized samples (Chen et al., 1987; Choi et al., 2003). Ninhydrin is a reagent commonly used to determine peptides using this difference approach; however the ninhydrin assay requires several minutes to produce significant color with AA and 15 to 30 min at 100°C to yield maximal absorbance (Greenstein and Winitz, 1961). Moreover, ninhydrin has also been reported to give a relatively high interference from ammonia (Broderick and Kang, 1980; Winters et al., 2002), which is a degradation end product in the IIV method. Church et al. (1983) developed an *o*-phthaldialdehyde (**OPA**) colorimetric assay based on absorption at 340 nm, which was a rapid, convenient, and sensitive procedure for determination of proteolysis in milk. The use in the IIV system of this assay, adapted to flow injection analysis (**FIA**), could decrease analysis time and improve the accuracy of results by measuring the peptides produced during protein degradation.

The aim of the present work was to quantify the contribution of oligopeptide formation in the Michaelis-Menten IIV system to ruminal protein degradation rate and RUP estimates in protein concentrates by comparing results obtained with the OPA colorimetric (**OPA-C**) assay versus the OPA fluorimetric (**OPA-F**) assay. Initial work was conducted on casein and samples of soybean meal. The method was then extended to more slowly degraded proteins (blood meal and corn gluten meal) and other soybean meals.

MATERIALS AND METHODS

Donor Animals and Diets

Inoculum was obtained from 2 lactating Holstein-Friesian donor cows surgically fitted with ruminal cannulas (Bar Diamond; Parma, ID). Cows were fed a diet containing alfalfa and corn silages plus a concentrate mixture of rolled high moisture corn, soybean meal, and vitamins and minerals. The diet averaged 16.5% CP and 1.60 Mcal of NE_L/kg of DM (computed at 3× maintenance; NRC, 2001). Surgical care and general maintenance of the animals was conducted as outlined by the guidelines of the University of Wisconsin institutional animal care and use committee.

Protein Samples

Studies were initially conducted using only 4 standard proteins: 1 sample of casein (no. C-5890, Sigma Chemical Co., St. Louis, MO), 2 samples of solvent-extracted soybean meal (**SSBM1** and **SSBM2**), and 1 sample of expeller-extracted soybean meal (**ESBM**). The 2 SSBM were selected from several samples to represent a range of soluble-N contents. In this phase, the effect of using different N levels on estimated rates was tested in incubations with a maximum of 100 tubes in each run. Corn gluten meal (**CGM**), blood meal (**BM**), and 2 other samples of solvent soybean meal (**SSBM3** and **SSBM4**) were studied in later incubations.

Samples were ground using a laboratory mill fitted with a 1-mm screen (Wiley mill, Arthur H. Thomas Co., Philadelphia, PA). All samples were analyzed for DM (AOAC, 1980) and total N by combustion assay (Leco FP-2000 N Analyzer; Leco Instruments, Inc., St. Joseph, MI) before the in vitro incubations.

In Vitro Protocol

Incubations were conducted in 50-mL centrifuge tubes fitted with stoppers with Bunsen valves. The 4 standard proteins were weighed to the nearest 0.1 mg into duplicate tubes in amounts equivalent to 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 10.0, 12.0, 16.0, 24.0, and 30.0 mg of N/tube. The 4 other protein samples were weighed in amounts equivalent to: 2.0, 4.0, 6.0, 12.0, 16, and 30 mg of N/tube and analyzed in separate incubations.

Whole ruminal contents were collected from beneath the fibrous mat in the rumen and filtered at the barn

through 2 layers of cheesecloth. The filtrates were quickly transported to the laboratory and then filtered again through 4 layers of cheesecloth; equal volumes of strained rumen fluid from each cow were mixed together. Per liter of mixed strained rumen fluid, 8 g of maltose (Sigma no. M-2250, Sigma Chemical Co.), 4 g of xylose (Sigma no. X-1500), 4 g of soluble starch (Sigma no. S-2004), and 2.5 g of $NaHCO_3$, dissolved in 150 mL of distilled water, plus 4 g of citrus pectin (Sigma no. P-9135), dissolved with heating and slow stirring into 150 mL of McDougall buffer (McDougall, 1948), were added. Then, 0.2 mL of the surfactant Antifoam 204 (Sigma no. A-6426) was added and the inoculum preincubated at 39° for 3 h under a CO_2 flow. Every hour, the pH was measured; if the pH was ≤6.2, the pH was adjusted to 6.4 by slow addition of 3 *N* NaOH. Inhibitor solutions (Broderick, 1987) were prepared by dissolving with stirring 0.267 g of hydrazine sulfate (HS; Sigma no. H-7394) in a 35-mL aliquot of McDougall buffer and 0.062 g of chloramphenicol (CAP; Sigma no. C-0378) in a second 35-mL aliquot of McDougall buffer. Addition of these 2 solutions to the 1,300 mL of preincubated medium yielded 1,370 mL of inoculum containing 1.5 m*M* HS and 45 mg of CAP/L. Mercaptoethanol (ME; Sigma no. M-6250) was then added (0.321 mL) as a reducing agent to give 3 m*M* ME in the inoculum.

During the time the inoculum was preincubating, 5 mL of warm (39°C) McDougall buffer was added to each incubation tube to suspend all proteins in buffer for 1 h before the start of the incubation. Incubations were begun by dispensing 10 mL of inoculum/tube; immediately after inoculation, tube headspace was flushed with CO_2, tubes were capped, swirled, and incubated for 2 h at 39°C (±0.5) in a warm room under continuous mixing with a wrist-arm shaker (set at 100 cycles/min). At the end of the incubation, 1.25 mL (65% wt/vol) of TCA was added to each tube and tubes were placed on ice. After 30 min, about 4 mL of sample from each incubation tube was decanted into a 12×75-mm plastic tube and tubes centrifuged (15 min, 14,000 × *g* at 4°C). Supernatants were then transferred to clean tubes, which were stored at 4°C until analyzed the following day as described below. Duplicate blank tubes bracketed each 44 protein-containing tubes (22 tubes/protein source) and typical incubations used about 100 tubes. Two preliminary runs were conducted with casein and ESBM only, the proteins with the highest and lowest degradation rates, to develop an operating protocol. All other incubations were replicated 3 times with all 4 standard proteins and 2 times with the other 4 protein sources.

Chemical Analyses

Proteins were analyzed for buffer-soluble N (**BSN**) using the method of Licitra et al. (1996) with the following modifications: McDougall buffer was used for extractions, extracts were filtered through Whatman no. 41 ashless glass filters, and total insoluble N was analyzed by the combustion (Leco FP-2000; Leco Instruments, Inc., St. Joseph, MI) of the filter plus the retained residue.

The proportion of total N already degraded at $t = 0$ (**FD0**) was determined on all proteins by weighing 4 replicates of 100 mg of each protein into 50-mL centrifuge tubes and then adding 15 mL of warm (39°C) McDougall buffer. Samples were swirled and incubated with shaking in the warm room (39°C ± 0.5) for 2 h. At the end of this buffer-only incubation, 1.25 mL (65% wt/vol) of TCA was added and samples were processed as described previously for the in vitro incubations. The total AA (**TAA**) contents of proteins (μmol/mg of N) were determined by hydrolyzing 4 replicates of each protein in 6 *N* HCl for 24 h at 105°C under an N_2 atmosphere, using a ratio of 1 mg sample of N/5 mL of acid (Block and Weiss, 1956). After hydrolysis, samples were cooled, diluted with distilled water, HCl was removed by vacuum evaporation (Savant SC110 Speedvac Concentrator, Savant Instruments, Inc., Farmingdale, NY), and the residues were redissolved in distilled water and re-evaporated. Residues were then dissolved in 4 mL of distilled water and stored (−20°C) for later analysis.

Protein hydrolysates and TCA supernatants (from in vitro incubations and from FD0 extractions) were analyzed for ammonia by a phenol-hypochlorite assay adapted to FIA (Lachat Quick-Chem 8000 FIA; Zellweger Analytical, Milwaukee, WI). The OPA reaction (Roth, 1971), based on absorbance at 340 nm (**OPA-C**; TAA + oligopeptides) or on fluorescence at 450 nm (**OPA-F**; TAA), was used on the same samples to analyze concentrations of TAA by flow injection analyzer (Lachat Quick-Chem 8000 FIA; Zellweger Analytical) modified with a UV light source (AIS D-1000; Analytical Instrument Systems, Flemington, NJ) for absorbance at 340 nm (OPA-C assay) and interfaced with a fluorimeter (FS-950 Fluoromat; Kratos Analytical Instruments, Westwood, NJ) for fluorescence at 450 nm (OPA-F assay).

Solutions were prepared in distilled water containing 1 m*M* of the 20 common protein AA, 13 dipeptides, and 14 tripeptides and oligopeptides; all AA and peptides were obtained from Sigma-Aldrich Co. (St. Louis, MO). Leucine standards encompassing the concentration

ranges of all unknown samples were used in both OPA assays.

Computation of Rate and Extent of Degradation

Extent of protein degradation ($\mathbf{S_D}$, mg of N/mL of inoculum) was computed for each protein source at each level of N addition from net release of N as ammonia plus TAA determined using either the OPA-F or the OPA-C assay with the equation

$$S_D = \{([NH3]_{prot} - [NH3]_{blank}) \times 0.0140067 + ([TAA]_{prot} - [TAA]_{blank})/(TAA/N)\} \times 16.25/10,$$

where $[NH3]_{prot}$, $[NH3]_{blank}$, $[TAA]_{prot}$, and $[TAA]_{blank}$ are concentrations of ammonia and TAA (μmol/mL) determined by either the OPA-F or OPA-C assay in, respectively, protein-containing and blank incubation tubes; 0.0140067 is the ammonia-N constant (mg of N/μmol of NH_3); TAA/N is the ratio of TAA (determined by acid hydrolysis of test proteins using either the OPA-F or OPA-C assay) to total N (μmol/mg of N); 16.25 (15 + 1.25) is the total tube volume (mL); and 10 is the volume (mL) of added inoculum. Velocity of protein degradation [**v**, mg of N/(mL of inoculum · h)], was computed for each protein source at each level of N by dividing S_D, computed using either TAA assay, by incubation time, t (2 h):

$$v = S_D/2.$$

Protein N remaining undegraded at the end of the incubation for each amount of added N ($\mathbf{S_t}$, mg of N/mL of inoculum), was calculated as the difference between the amount of N added at t = 0 divided by the volume of inoculum ($\mathbf{S_0}$, mg of N/mL of inoculum) and the S_D:

$$S_t = S_0 - S_D.$$

These data were used to estimate fractional degradation rate ($\mathbf{k_d}$) from the tangent through the origin of the velocity versus substrate concentration ($[S_0]$) curve, and determined from the ratio of maximum velocity (Vmax) to Michaelis constant ($\mathbf{K_m}$; i.e., k_d = Vmax/K_m; Mahler and Cordes, 1966). This was considered to be a blended initial rate representing a composite of the rates of ruminal degradation of all protein fractions within a sample. This rate was determined using the integrated Michaelis-Menten model (Segal, 1976), as described earlier (Broderick and Clayton, 1992):

$$S_t = S_0 - t \times k_d \times K_m + K_m \times \ln(S_0/S_t),$$

where S_0 and S_t (mg of N/mL of inoculum) are as defined above, t (h) is the duration of incubation (2 h), and K_m (mg of N/mL of inoculum) is the Michaelis constant defined as that concentration of substrate that gives half-maximal velocity. The parameters K_m and k_d were estimated by nonlinear regression (NLIN) using SAS (SAS Institute, 1999–2000). The k_d values were then adjusted (Ak_d) for FD0 as follows:

$$Ak_d = k_d - FD0/t,$$

where t = 2 h. The proportion of RUP was computed using the model of Waldo et al. (1972), applying the blended rate philosophy and different rates of passage for the insoluble (k_{pi}) and soluble (k_{ps}) N fractions:

$$RUP = [(Total\ N - BSN) \times k_{pi}/(k_{pi} + Ak_d)] + [(BSN - FD0) \times k_{ps}/(k_{ps} + Ak_d)],$$

where k_{pi} was set equal to 0.06/h and k_{ps} was set equal to 0.16/h. No correction was made for ADIN content because of very low or 0 concentrations in all 8 proteins.

Statistical Analysis

Statistical analysis was carried out using the mixed procedure of SAS (SAS Institute, 1999–2000). Ruminal degradation rates observed for casein and ESBM in the 2 preliminary runs were not different ($P = 0.34$) from the rates obtained in the subsequent 3 incubations and all 5 observations were used for both proteins. Thus, for standard protein samples, n = 5 for casein and ESBM and n = 3 for SSBM1 and SSBM2; n = 2 for the other 4 protein sources. The model included protein, method (OPA-F vs. OPA-C) and protein × method interaction as fixed effects and run as a random effect. Least squares means estimates are reported; separation of least squares means was conducted at $\alpha = 0.05$ using the PDIFF option in the LSMEANS statement. For all statistical analyses, significance was declared at $P \leq 0.05$ and trends at $P \leq 0.10$.

RESULTS AND DISCUSSION

Response of AA and Peptides in OPA Assays

Typical standard curves obtained with the OPA assays are in Figure 1. Lack of apparent curvilinearity of

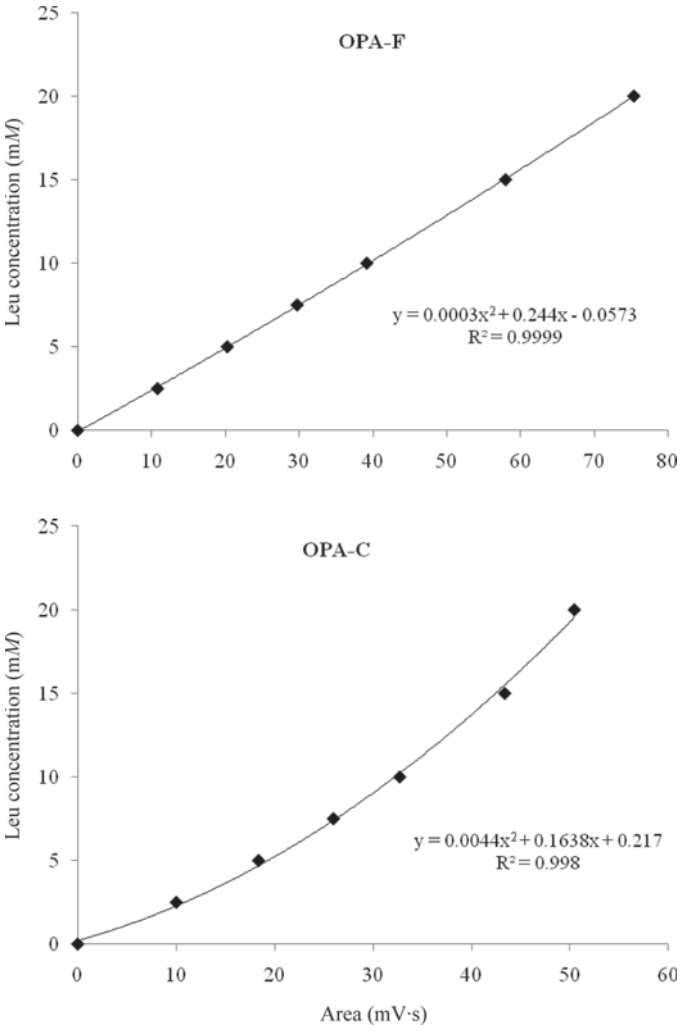

Figure 1. Standard curves for the *o*-phthaldialdehyde fluorimetric (OPA-F) and *o*-phthaldialdehyde colorimetric (OPA-C) assays using Leu as the calibration standard.

the OPA-F standard curve reflects the wider dynamic range of fluorimetric than colorimetric assays; however, both assays yielded satisfactory calibration curves with excellent r^2 values with up to 20 m*M* Leu.

Relative molar fluorescence and absorbance responses of 20 protein AA are in Table 1. The OPA forms adduct only with primary amino groups (Böhlen and Mellet, 1979); thus, response with the secondary AA Pro was nil in both assays. Relative OPA-C response for Lys was 2.35. Lysine has 2 amino groups that react with OPA, which enhances its absorbance at 340 nm. Relative OPA-F response of Lys was only 0.67; lack of intense fluorescence is due to interaction of the 2 isoindole groups in the di-substituted product that is formed in the reaction between Lys and OPA (Chen et al., 1979).

One of the main advantages of using the OPA assay is the rapid velocity of the reaction: free AA and peptides react with OPA at rates of 0.05 to 0.07/s at ambient temperature (Church et al., 1985), reaching maximal response in approximately 1 min (Goodno et al., 1981). Total AA content can also be determined by ninhydrin assay but ninhydrin requires several minutes to produce significant color with AA and 15 to 30 min at 100°C to yield maximal absorbance with the 20 protein AA (Greenstein and Winitz, 1961). Overall, the OPA methods adapted to FIA allowed analysis of 90 samples/h, thus, significantly decreasing analysis time in comparison with the ninhydrin assay (Broderick and Kang, 1980). Further, ninhydrin also gives rise to relatively high response to ammonia (Broderick and Kang, 1980; Winters et al., 2002), which is always present in ruminal inocula and which would contribute to interference in assaying extent of degradation using the IIV method.

The average relative responses (Leu = 1.0) for dipeptides and for tri- and oligopeptides are in Table 2. Low fluorescence yields from OPA-peptide adducts are due to quenching of fluorescence by the peptide bond (Chen et al., 1979); consequently, OPA produces significant fluorescent yield only from free AA or from peptides containing unreacted Lys residues. High 340-nm absorbance from OPA-peptide adducts confirms the results of Church et al. (1983) who measured proteolysis in milk and adapted this procedure to quantify the extent of protein breakdown in the presence of several different proteases with satisfactory results (Church et al., 1985). Compared with the OPA-C assay, use of the OPA-F assay to quantify protein breakdown products could underestimate extent of proteolysis due to very low contributions from peptides.

Determination of In Vitro Ruminal Protein Degradation Rates and RUP

Data on N-composition of the standard proteins are in Table 3. As well as having the greatest N content, casein had the highest proportion of soluble N. Total N content was similar among the 3 soybean meals, whereas soluble N was lowest for ESBM, intermediate for SSBM1, and highest for SSBM2. The TAA value (μmol/mg of N), determined after HCl hydrolysis, was greater ($P < 0.001$) using the OPA-C assay versus the OPA-F assay, reflecting the differing responses of individual AA in each assay. Samples were not analyzed for individual AA composition; however, using an assumed standard AA composition for soybean meal (American Soybean Association and United Soybean Board, 2000) and applying the individual AA responses reported in Table 1, relative total AA content (μmol/mg of N)

Table 1. Relative molar response (Leu = 1.0) of protein AA in the colorimetric (OPA-C) and fluorimetric (OPA-F) *o*-phthaldialdehyde assays adapted to flow-injection analysis

Item	OPA-C	OPA-F
Protein AA		
Ala	0.904	0.893
Arg	1.127	0.959
Asp	0.508	0.674
Asn	0.839	0.796
1/2-Cys	1.044	0.031
Glu	0.763	0.846
Gln	1.003	0.909
Gly	1.301	0.873
His	0.998	1.039
Ile	0.778	0.944
Leu	1.000	1.000
Lys	2.347	0.670
Met	1.143	1.040
Phe	0.932	1.001
Pro	0.008	0.000
Ser	1.079	0.982
Thr	0.614	0.725
Trp	0.992	1.038
Tyr	0.892	0.928
Val	0.722	0.879
Mean	0.950	0.811
SD	0.430	0.295

would have been 53 and 44, determined using, respectively, the OPA-C and OPA-F assays.

Protein already degraded at t = 0, fraction FD0, is NPN constituted by ammonia, free AA, and small peptides. This fraction was considered to be already degraded N that would be largely utilized for growth or converted to ammonia by ruminal microorganisms. The FD0 fractions in the standard proteins were composed almost totally of free AA and peptides with virtually no ammonia (Table 3). Degradability of the FD0 fraction in the rumen should, theoretically, be comparable with that proposed by the NRC (2001) model for protein fraction A. However, unlike fraction FD0, NRC fraction A also includes soluble intact protein and protein associated with small particles, which appear to have degradation rates similar to proteins associated with large particles that do not wash out of the in situ bag (Gierus et al., 2005). Wide variability exists among feeds in fraction A values: Olaisen et al. (2003) reported wash-out CP fractions (% of total CP) ranging from 10% for corn gluten meal to 67.6% for pea grains; as a consequence, in situ degradation estimates are highly dependent on soluble-N content of protein sources. Hence, in situ measurements for concentrate feeds, such as peas and lupines, with high levels of buffer-soluble N would have limited value (Huhtanen et al., 2008). Similarly, fractions A (% of total CP) reported by NRC (2001) for expeller soybean meal (8.7%) and solvent soybean meal (22.5%) are much higher than the FD0 observed in our study, and the assumption that this fraction is completely degraded in the rumen likely would result in erroneous evaluations of these feedstuffs. A recent meta-analysis (Huhtanen and Hristov, 2009) showed that ruminal degradability of dietary protein, as predicted by NRC (2001), was not a significant factor in predicting milk protein yield and milk N efficiency in dairy cows. Although soluble protein-N appears to be more readily degraded in the rumen (Krishnamoorthy et al., 1983; Volden et al., 2002), N solubility did not give reliable predictions of in vivo protein degradation of feedstuffs (Nocek et al., 1979; Stern and Satter, 1984). Grabber and Coblentz (2009) reported inconsistencies between protein fractionation based on N solubility and biologically based assays (in situ and protease methods), which resulted in conflicting estimates of ruminal protein degradability; this highlighted the need for complementary data on CP pool sizes and protein degradation rates measured using ruminal inocula. Furthermore, Huhtanen et al. (2008) suggested that determining soluble N in silages was of limited value because the faster ruminal passage rate of the liquid phase compensated, at least partly,

Table 2. Relative molar response (Leu = 1.0) of selected di-, tri-, and oligopeptides in the colorimetric (OPA-C) and fluorimetric (OPA-F) *o*-phthaldialdehyde assays adapted to flow-injection analysis

Item	OPA-C	OPA-F
Dipeptide		
Ala_2	1.277	0.175
D-Ala-L-Leu	1.152	0.238
AlaGly	1.173	0.161
AlaSer	1.241	0.136
AlaPhe	1.176	0.087
Gly_2	1.225	0.281
Gly-D-Ala	1.301	0.316
Gly-D-Leu	1.316	0.253
LeuGly	1.100	0.148
LeuLeu	0.633	0.080
MetAla	0.945	0.085
PheGly	0.417	0.038
Phe_2	0.566	0.043
Mean	1.040	0.157
SD	0.305	0.091
Tri- and oligopeptides		
Ala_3	0.936	0.085
Ala_4	0.989	0.087
$AlaGly_2$	0.926	0.062
ArgLysAspValTyr	1.842	0.284
AspSerAspProArg	0.453	0.038
Gly_3	1.170	0.138
Gly_2-D-Leu	1.097	0.131
Gly_2Phe	1.085	0.141
Gly_2TyrArg	1.127	0.119
Gly_2Val	1.049	0.117
Gly-D,L-Leu-D,L-Ala	1.086	0.101
$GlyProGly_2$	1.223	0.165
$LeuGly_2$	0.707	0.046
MetAlaSer	0.586	0.035
Mean	1.020	0.111
SD	0.327	0.064

Table 3. Composition of standard protein sources

Protein[1]	Total N (% DM)	Soluble N (% of total N)	Free AA (% of total N)	Oligopeptides (% of total N)	OPA-C TAA[2] (μmol/mg of N)	OPA-F TAA[3] (μmol/mg of N)
Casein	14.23	99.9	0.27	0.34	50	40
ESBM	6.80	4.5	0.53	1.38	50	40
SSBM1	7.22	17.3	0.76	1.28	51	40
SSBM2	7.36	37.4	1.04	1.20	55	43

[1]ESBM = expeller-extracted soybean meal; SSBM = solvent-extracted soybean meal.
[2]Free AA content determined with the *o*-phthaldialdehyde colorimetric (OPA-C) assay after hydrolysis in 6 *N* HCl. TAA = total AA.
[3]Free AA content determined with the *o*-phthaldialdehyde fluorimetric (OPA-F) assay after hydrolysis in 6 *N* HCl.

for differences in the degradation rates of soluble and insoluble N.

Ruminal degradation rates and RUP values obtained using the OPA-C and OPA-F assays are reported in Table 4. The OPA-C assay gave significantly higher degradation rates than did the OPA-F assay ($P = 0.015$), with the difference due to the detection of oligopeptides released during protein breakdown. The trend for an interaction between sample and method ($P = 0.082$) may be due to more rapidly degraded protein (casein) giving rise to larger amounts of peptides. There is reason to consider oligopeptides as protein that is already degraded. Depardon et al. (1995) indicated that high-molecular-weight peptides were more rapidly broken down relative to intermediate-sized peptides and to small assimilable peptides; thus, peptides detected in the OPA-C assay, when added to ammonia-N, would appear to be a more reliable way to quantify the main protein breakdown products.

Degradation rates for SSBM and ESBM obtained in vitro by the OPA-C assay were 2.5 (SSBM 1), 1.9 (SSBM 2), and 3.6 (ESBM) times more rapid than in situ estimates reported by NRC (2001). Degradation rates for SSBM and ESBM measured in vivo by Reynal and Broderick (2003) were 2.4 and 3.2 times higher than estimates reported by NRC (2001), with in vivo RUP values of 27 and 45% for SSBM and ESBM, respectively, and similar to results obtained in the present study. A significant effect of protein source ($P < 0.001$) on K_m and a tendency for an effect of assay ($P = 0.058$) were found. The K_m is the concentration of substrate required to produce half of the maximal reaction velocity and represents the affinity of an enzyme for a substrate; lower K_m values indicate higher affinities of the enzyme system for the substrate (Segal, 1976). In the current study, ESBM had the highest K_m values using both assays and, presumably, the lowest affinity for microbial degradative enzymes.

Table 4. Effect of the method on Michaelis constant (K_m), degradation rates, adjusted rates, and RUP values for standard proteins

Method[1]	Protein[2]	K_m (mg of N/mL of SRF[3])	k_d[4] (per h)	Ak_d[5] (per h)	RUP (% of CP)
OPA-C	Overall	0.776	0.280	0.272	30.5
	Casein	0.293^{b}	0.593^{a}	0.590^{a}	21.3^{c}
	ESBM	1.604^{a}	0.096^{c}	0.086^{c}	41.8^{a}
	SSBM1	0.559^{b}	0.241^{b}	0.231^{b}	24.4^{c}
	SSBM2	0.648^{b}	0.192^{b}	0.181^{b}	34.6^{b}
OPA-F	Overall	0.541	0.219	0.215	35.2
	Casein	0.195^{b}	0.445^{a}	0.444^{a}	26.7^{c}
	ESBM	1.093^{a}	0.075^{c}	0.072^{c}	47.5^{a}
	SSBM1	0.361^{b}	0.192^{b}	0.188^{b}	28.6^{c}
	SSBM2	0.513^{b}	0.162^{bc}	0.157^{bc}	38.0^{b}
	SEM	0.106	0.038	0.022	1.57
Probability	Protein	<0.001	<0.001	<0.001	<0.001
	Method	0.058	0.009	0.015	0.002
	Protein × method	0.498	0.100	0.082	0.910

[a–c]LSM within column and method (excluding overall means) with different superscripts are different ($P < 0.05$).
[1]OPA-C = *o*-phthaldialdehyde colorimetric assay; OPA-F = *o*-phthaldialdehyde fluorimetric assay; Probability = probability of significant effects of protein, method, and the protein-by-method interaction.
[2]ESBM = expeller-extracted soybean meal; SSBM = solvent-extracted soybean meal.
[3]Strained ruminal fluid.
[4]Degradation rate.
[5]Adjusted degradation rate.

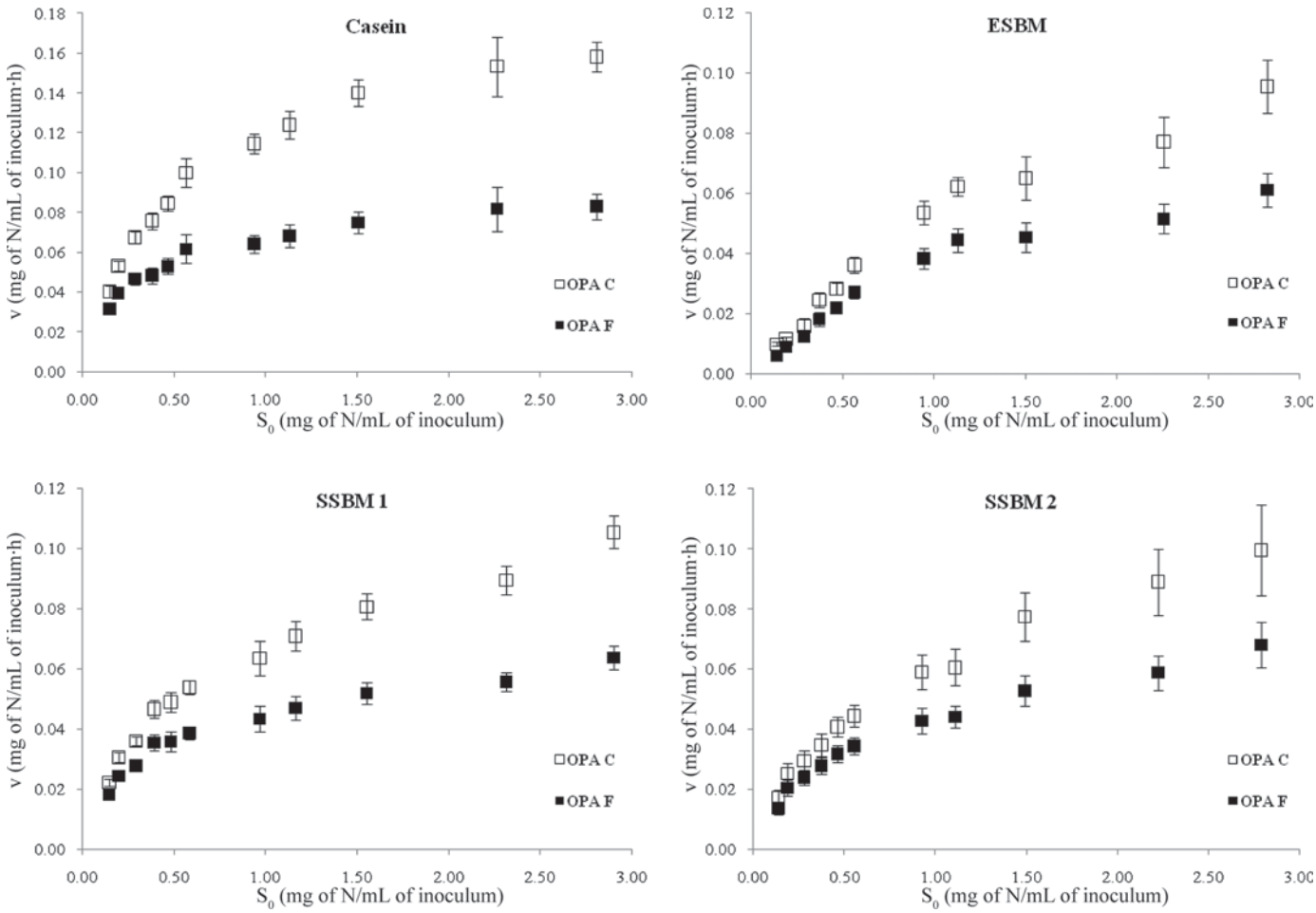

Figure 2. Velocity of degradation (v) as a function of substrate concentration (S_0) determined using the *o*-phthaldialdehyde colorimetric (OPA-C) and *o*-phthaldialdehyde fluorimetric (OPA-F) assays for the 4 standard proteins. ESBM = expeller-extracted soybean meal; SSBM = solvent-extracted soybean meal.

Plots of velocity of degradation as a function of substrate concentration for all 4 standard proteins are shown in Figure 2. Substantial variation in degradability among runs was present. Proteolytic activity of ruminal fluid has been shown to be highly affected by the type of diet (Siddons and Paradine, 1981; Broderick et al., 1991) and by animal (Wallace and Cotta, 1988). Ahvenjärvi et al. (2009) reported a coefficient of variation of 46.7% for protein degradation rate determined in vitro using ruminal inoculum from cows consuming similar amounts of the same diet. In our study, coefficients of variation for degradation rate and RUP were, respectively, 22.1 and 16.3% with the OPA-C assay and 21.5 and 14.7% with the OPA-F assay.

The regression forced through zero of degradation rates obtained using the OPA-C assay on rates obtained with the OPA-F assay for the 4 standard proteins is in Figure 3a. The regression had a satisfactory R^2 (0.937) and a slope of 1.30, reflecting the more rapid rates observed with the OPA-C method due to oligopeptide detection. The regression without casein, the protein characterized by the highest peptide formation rate, is in Figure 3b. That slope was lower and equal to 1.25, but with a similar coefficient of determination.

The protein degradation rates for the 4 other protein concentrates are in Table 5. Again, adjusted rates were higher with the OPA-C versus the OPA-F method ($P < 0.021$). The BM and CGM proteins had the lowest degradation rates and the rate for BM was the same by both the OPA-C and the OPA-F assays, indicating very little or no accumulation of oligopeptides. The trend ($P = 0.052$) for an interaction between sample and assay method may be due to more slowly degraded proteins giving rise to smaller amounts of peptides. Degradation rates measured by the OPA-C assay were different for SSBM3 and SSBM4, whereas the OPA-F rates did not differ between these 2 proteins. The degradation rate of CGM protein, determined by the IIV method

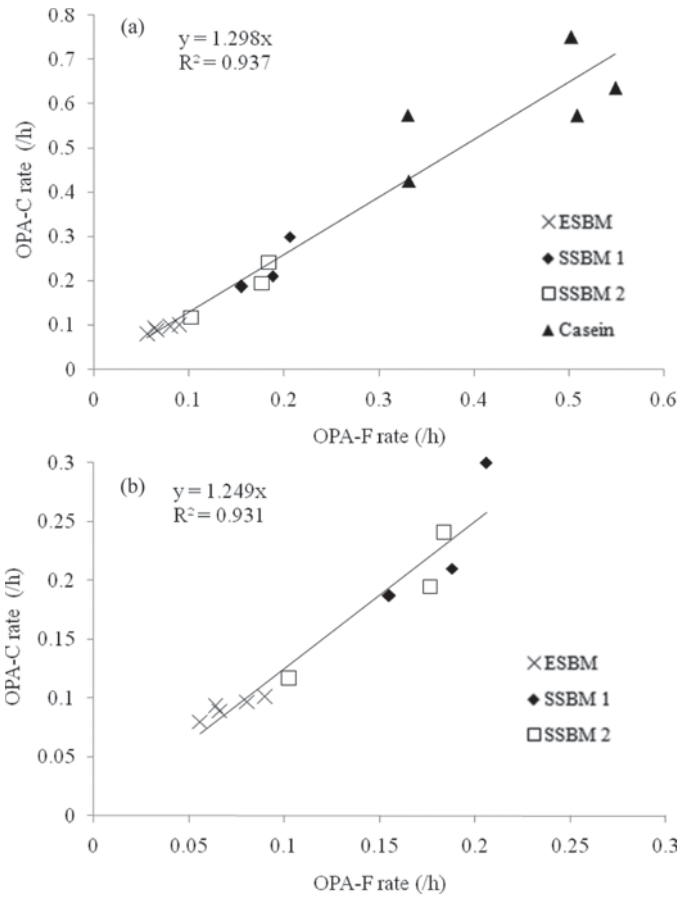

Figure 3. Relationship between the degradation rates determined with the *o*-phthaldialdehyde colorimetric (OPA-C) and the *o*-phthaldialdehyde fluorimetric (OPA-F) assays for all 4 proteins (a) and for the 3 soybean meals only (b). ESBM = expeller-extracted soybean meal; SSBM = solvent-extracted soybean meal.

using substrate-limiting amounts and OPA-F (Reynal and Broderick, 2003), was lower than the rate obtained in the present study with both of the assays. Overall, RUP content was lower with the OPA-C assay versus the OPA-F assay ($P < 0.025$). Blood meal RUP content was higher than the in vivo value reported by Reynal and Broderick (2003), whereas for CGM, the value was closer to the in vivo result. The RUP contents of SSBM were slightly lower than NRC (2001) tabulated value.

The protein degradation rates estimated in this work were assumed to reflect blended rates, which derived from proportionate contributions from all protein fractions in each source. Degradation rates for the total proteins in SSBM and ESBM were not significantly different from the rates for the residual buffer-insoluble proteins (Broderick and Clayton, 1992); thus, it appears that degradation rates estimated using the Michaelis-Menten approach will not be skewed by rapidly degraded fractions, and rates obtained for feed proteins are representative of degradation of the feedstuff as a whole. The different protein fractions from which these rates originate would not matter if all protein fractions passed out of the rumen at the same or similar rates. However, it is likely that at least 2 fractions would exist, soluble and insoluble proteins, which would be expected to flow at 2 different rates. We computed overall protein escape by applying a single-blended rate to both the soluble and insoluble fractions, using assumed passage rates of 0.16/h and 0.06/h. The theoretical data in Table 6 confirm that this is an acceptable approximation. For example, if a protein source similar to SSBM contained 25% soluble protein with k_d

Table 5. Protein degradation rates and RUP estimates for 4 other protein concentrates determined using the colorimetric (OPA-C) and fluorimetric (OPA-F) *o*-phthaldialdehyde assays in the inhibitor in vitro (IIV) method

Method	Protein[1]	N (% DM)	K_m[2] (mg of N/mL of SRF[3])	k_d[4] (per h)	Ak_d[5] (per h)	RUP (% of CP)
OPA-C	SSBM3	7.85	0.674[b]	0.214[a]	0.203[a]	27.7[c]
	SSBM4	8.72	1.062[b]	0.157[b]	0.142[b]	32.7[c]
	BM	15.77	2.461[a]	0.013[c]	0.012[c]	83.8[a]
	CGM	10.33	2.775[a]	0.038[c]	0.026[c]	69.6[b]
OPA-F	SSBM3	7.85	0.557[b]	0.146[a]	0.139[a]	35.8[c]
	SSBM4	8.72	0.722[b]	0.129[a]	0.121[a]	36.9[c]
	BM	15.77	1.636[a]	0.013[b]	0.012[b]	84.0[a]
	CGM	10.33	2.121[a]	0.029[b]	0.022[b]	73.4[b]
	SEM		0.227	0.012	0.012	2.32
Probability[6]	Protein		<0.001	<0.001	<0.001	<0.001
	Method		0.011	0.012	0.021	0.025
	Protein × method		0.294	0.054	0.052	0.348

[a–c]LSM within column and method with different superscripts are different ($P < 0.05$).

[1]SSBM = solvent-extracted soybean meal; BM = blood meal; CGM = corn gluten meal.

[2]Michaelis constant.

[3]Strained ruminal fluid.

[4]k_d = degradation rate.

[5]Ak_d = adjusted degradation rate.

[6]Probability of significant effects of protein, method, and the protein-by-method interaction.

Table 6. Theoretical computations of the effect of using blended degradation rates on RUP estimates

Protein[1]	Fraction	Proportion (%)	k_p[2] (per h)	Individual k_d[3] (per h)	Blended k_d (per h)	Escape of fraction (%)	RUP (%)
SSBM	Soluble	25	0.16	0.30		8.7	30.1
	Insoluble	75	0.06	0.15		21.4	
	Soluble	25	0.16		0.1875	11.5	29.7
	Insoluble	75	0.06		0.1875	18.2	
ESBM	Soluble	10	0.16	0.15		5.2	54.3
	Insoluble	90	0.06	0.05		49.1	
	Soluble	10	0.16		0.06	7.3	52.3
	Insoluble	90	0.06		0.06	45.0	

[1]SSBM = solvent-extracted soybean meal; ESBM = expeller-extracted soybean meal.

[2]Passage rate.

[3]Degradation rate.

= 0.30/h and 75% insoluble protein with k_d = 0.15/h, the computed protein escape from the respective fractions would be 8.7 and 21.4%, or a total of 30.1%. A blended rate may be computed by proportioning the rates over each fraction: blended rate = 0.25 × 0.30/h + 0.75 × 0.15/h = 0.1875/h. Applying this blended rate to each fraction and its corresponding passage rate would yield contributions of 11.5 and 18.2%, for an estimated total escape of 29.7%, a result only slightly different from 30.1%. A similar exercise with a protein source more similar to ESBM yielded a comparably similar result (Table 6). It is noted that, although the estimate of total escape differs little, contribution from soluble and insoluble fractions is more different. This would be of concern in vivo if protein fractions differed substantially in essential AA pattern.

CONCLUSIONS

The OPA colorimetric assay appears to be a simple, fast and reliable method to quantify small peptides. Applying this assay in the IIV method, using Michaelis-Menten saturation kinetics, yielded higher protein degradation rates than applying the OPA fluorescent assay, with the difference presumably due to the detection of oligopeptides released during protein breakdown. Both assays yielded degradation rates that were more rapid than the rates observed using in situ methods. The equation proposed to predict RUP, using blended rates and applying separate passage rates for soluble and insoluble N fractions, obviates a major limitation of the in situ model, which supposes that all soluble protein is completely degraded in the rumen. Further, degradation rates for typical feed proteins in the in vivo rumen may be assumed to be blended rates resulting from the proportionate degradation of all protein fractions. Applying the OPA colorimetric assay in conjunction with IIV Michaelis-Menten saturation kinetics is an approach that can be routinely used to estimate RUP on common concentrate feedstuffs. Further validation of the method on forage proteins and more complex concentrate mixtures is required; however, Colombini et al. (2010) have already reported preliminary estimates of protein degradation rates in tropical grasses obtained using the present approaches.

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