Characterization of the Proteolytic Activity of Commercial Proteases and Strained Ruminal Fluid[1,2]

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ABSTRACT: The objective of this research was to formulate a mixture of commercial proteases that would mimic the rate and extent of protein degradation obtained using strained ruminal fluid. The proteolytic activity of strained ruminal fluid and several commercial proteases was characterized using 13 L-amino acid *p*-nitroanilides as artificial substrates. A mixture of *Streptomyces griseus* protease, chymotrypsin, and proteinase K at .042, 2.5, and .5 enzyme units/mL, respectively, was similar to the activity of strained ruminal fluid against the same artificial substrates. However, degradative activities were different in incubations with feed proteins as substrates. The rates of degradation of expeller soybean meal, solvent soybean meal, and casein were .08, .05, and .08/h, respectively, using the enzyme mixture and .03, .15, and .24/h using strained ruminal fluid. A second experiment compared degradative activity of *S. griseus* protease at .066 enzyme units/mL, ficin at .5 enzyme units/mL, and a mixture of trypsin, carboxypeptidase B, chymotrypsin, and carboxypeptidase A at 116.6, .5, 2.5, and .5 enzyme units/mL, respectively. Protein degradation rates obtained with strained ruminal fluid were two to six times faster than those obtained with the enzyme mixtures. A third experiment compared the degradability of 15 feed proteins with the mixture of trypsin, carboxypeptidase B, chymotrypsin and carboxypeptidase A to that with strained ruminal fluid. Degradation rates obtained using strained ruminal fluid ranged from .007 to .217/h; degradation rates using the enzyme mixture ranged from .010 to .079/h and were lower (P = .004) than with strained ruminal fluid. Overall, the experiments indicated that the commercial enzymes tested did not mimic the protein degradative activity of strained ruminal fluid.

Key Words: Protease, Proteolytic Activity, Ruminal Fluid, In Vitro

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Introduction

Methods to evaluate ruminal protein degradation can be classified as in vivo, in situ, and in vitro. In vivo methods are cumbersome, prone to error, and not conducive to routine use (Stern et al., 1994). The in situ method has poor repeatability between laboratories (Cottrill, 1993) and requires correction for the microbial contamination of the feed residues within the bags (Nocek, 1988). Some researchers (Broderick, 1987; Raab et al., 1983) used strained ruminal fluid (**SRF**) as the inoculum for in vitro incubations. However, the need for donor animals to provide SRF contributes to variation in inocula and limits applicability of in vitro methods to commercial laboratories. Others have used commercial proteases from sources other than ruminal fluid (Krishnamoorthy, 1982; Poos-Floyd et al., 1985); however, results obtained from incubations with such proteases may be misleading (Mahadevan et al., 1987). Nevertheless, a method for determining ruminal degradability of feed proteins based on commercially available enzymes would be advantageous because it would avoid the problems arising from use of SRF (Mahadevan et al., 1980). A mixture of commercial proteases with a broad range of specificities, added at appropriate concentrations, may have overall proteolytic activity similar to that of SRF. The objective of these experiments was to formulate a mixture of commercial proteases that would mimic the rates and extents of protein degradation obtained with SRF.

[1]Mention of a trademark or proprietary product in this paper does not constitute a guarantee or warranty of the product by the USDA or the ARS and does not imply its approval to the exclusion of other products that also may be suitable.

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Table 1. L-Amino acid-*p*-nitroanilides used as artificial substrates in incubations with strained ruminal fluid and commercial enzymes as inoculum sources

Substrate	Code[a]	Molecular weight
L-Valine-*p*-nitroanilide	L-Val	273.7
L-Lysine-*p*-nitroanilide	L-Lys	428.1
L-Alanine-*p*-nitroanilide	L-Ala	245.7
L-Methionine-*p*-nitroanilide	L-Met	269.3
L-Leucine-*p*-nitroanilide	L-Leu	287.7
L-Proline-*p*-nitroanilide	L-Pro	349.3
Glycine-*p*-nitroanilide	Gly	195.2
L-Arginine-*p*-nitroanilide	L-Arg	367.2
N-Benzoyl-L-tyrosine-*p*-nitroanilide	BTyr	405.4
N-Benzoyl-L-arginine-*p*-nitroanilide	BArg	434.9
N-Benzoyl-L-cysteine-*p*-nitroanilide	BCys	331.4
L-Alanine-alanine-*p*-nitroanilide	$L\text{-}Ala_2$	316.7
L-Alanine-alanine-alanine-*p*-nitroanilide	$L\text{-}Ala_3$	387.8

[a]Identification code used in Table 3 and Figure 1.

Materials and Methods

Experiment 1. Whole ruminal contents were obtained from a ruminally cannulated lactating dairy cow approximately 2 h after it was fed a diet of (g/kg DM) 600 alfalfa silage, 282 shelled cracked corn, 100 soybean meal, 11 dicalcium phosphate, and 7 trace mineral salt. The ruminal inocula for the in vitro assays were prepared by the method of Craig et al. (1984). The SRF was obtained by squeezing whole ruminal contents through two layers of cheesecloth to yield a given volume of SRF. To extract some of the particle-associated microorganisms, the remaining solids residue was washed four times using a total volume of pre-warmed (39°C) McDougall's buffer (1948) equal to the original volume of the SRF. The SRF plus buffer extract were mixed then filtered through eight layers of cheesecloth. Oxygen-free CO_2 (Hungate, 1969) was used to purge air from all vessels and in the preparation of McDougall's buffer.

The SRF inocula were incubated with 13 L-amino acid *p*-nitroanilides as substrates (Table 1). Substrates were diluted to 1.5 m*M* in a 50:50 solution (ethanol:50 m*M* potassium phosphate buffer); concentration of each *p*-nitroanilide in the final medium was .50 m*M*. Incubations were performed in 10-mL test tubes capped with rubber stoppers, after flushing with O_2-free CO_2, for 20 min in a 39°C water bath and replicated three times. Incubations with the same artificial substrates were conducted with proteolytic enzymes (Table 2). Based on results obtained from incubations with individual enzymes, a set of enzymes was selected and mixed to give a combined proteolytic activity similar to that of SRF. The enzymes (Table 2) were diluted with McDougall's buffer (39°C) in test tubes that were neither flushed with CO_2 nor capped during the 20-min incubation at 39°C. Following incubation with SRF or enzymes, hydrolysis of artificial substrates was determined at 546 nm (Sargent-Welch model 6-550 UV/VIS spectrophotometer, Skokie, IL) from *p*-nitroaniline release quantified using diazotization (Appel, 1974). Standard curves were prepared in each incubation using *p*-nitroaniline

Table 2. Sources and specificities of the commercial proteases tested as inoculum sources

Enzyme (source)	EC[a]	Specificity	pH optimum
Aspergillus oryzae (fungal)[c]	NA[b]	NA	7.0–8.0
Aspergillus sojae (fungal)[c]	NA	NA	7.0–8.0
Bacillus licheniformis (bacterial)[c]	NA	Nonspecific endopeptidase	7.0–8.0
Bacillus polymyxa (bacterial)[c]	3.4.24.4	Zn-Metalloenzyme, Nonspecific endopeptidase	7.0–9.0
Bacillus thermoproteolyticus (bacterial)[c]	NA	Zn-metalloprotease, Nonspecific endopeptidase	7.0–9.0
Bacillus subtilis (bacterial)[c]	3.4.21.14	Serine protease, Nonspecific endopeptidase	7.0–11.0
Bromelain (pineapple stem)[d]	3.4.22.4	Cysteine protease, Nonspecific endopeptidase	5.0–7.0
Chymotrypsin (bovine pancreas)[e]	3.4.21.1	Serine protease, Endopeptidase: Tyr, Phe and Trp	7.0–9.0
Elastase (porcine pancreas)[f]	3.4.21.36	Serine protease, Endopeptidase	8.8
Ficin (fig tree latex)[g]	3.4.22.3	Cysteine protease, Nonspecific endopeptidase	6.5
Papain (papaya latex)[h]	3.4.22.2	Cysteine protease, Endopeptidase	6.0–7.0
Streptomyces caespitosus (bacterial)[c]	NA	NA	7.0–8.0
Streptomyces griseus (bacterial)[c]	NA	Nonspecific exo- and endopeptidase	6.0–8.0
Proteinase K (*Tritirachium album*)[c]	3.4.21.14	Serine protease, Endopeptidase	7.5–10.5
Trypsin (bovine pancreas)[i]	3.4.21.4	Serine protease, Endopeptidase: Arg and Lys	8.0

[a]Enzyme commission number.
[b]Information not available.
[c]One unit hydrolyzes casein to produce color equivalent to 1.0 μmol/min of tyrosine at pH 7.5 at 35°C.
[d]One unit releases 1.0 μmol/min of *p*-nitrophenol from N-alpha-CBZ-L-lysine-*p*-nitrophenyl ester at pH 4.6 at 25°C.
[e]One unit hydrolyzes 1.0 μmol/min of BTEE at pH 7.8 at 25°C.
[f]One unit hydrolyzes 1.0 nanomol/sec of *p*-nitrophenol from N-t-BOC-L-alanine-*p*-nitrophenyl ester at pH 6.4.
[g]One unit produces a Δ A_{280} of 1.0/min at pH 7 at 37°C.
[h]One unit hydrolyzes 1.0 μmol/min of BAEE at pH 6.2 at 25°C.
[i]One BAEE unit = Δ A_{253} of .001/min with BAEE as substrate at pH 7.6 at 25°C.

solutions diluted in the 50:50 ethanol:buffer medium.

Enzymes also were incubated with casein, expeller soybean meal (**ESBM**), and solvent soybean meal (**SSBM**). Incubations were performed in 50-mL tubes; proteins were added at .125 mg N/mL (Broderick, 1987). The final volume was 15 mL; 5 mL of McDougall's buffer was added to each tube to soak the protein for 45 min prior to the addition of 10 mL of enzyme solution. Individual enzymes were diluted with warm (39°C) McDougall's buffer 30 min before incubation with the feedstuff. Enzyme mixtures were prepared no more than 5 min prior to addition to the protein. Incubations were conducted for 0 and 2 h in a warm room at 39°C; activity was stopped by placing the tubes in an ice water bath and adding 1.25 mL of 65% (wt/vol) trichloroacetic acid (Broderick, 1987). Tubes were held on ice for at least 30 min. An aliquot was transferred to a 12- × 75-mm disposable centrifuge tube and centrifuged for 20 min at 10,000 × *g* at 4°C. Supernatants were analyzed for total amino acids (**TAA**) by a semi-automated method (Broderick and Kang, 1980). Protein degradation rates and extents were estimated as computed by Broderick (1987), except that only the N in TAA was included in the equation. Rate and extent of degradation of casein, ESBM, and SSBM also were determined from net release of both NH_3 N and TAA N at 0 and 2 h using SRF in the inhibitor in vitro (**IIV**) method (Broderick, 1987).

Experiment 2. Three enzyme methods were compared with the IIV technique: *S. griseus* protease (Krishnamoorthy, 1982), ficin (Poos-Floyd et al., 1985), and a modification of an enzyme mixture designed to distinguish among heat-treated feedstuffs (Hansen et al., 1975). The feedstuffs selected for testing the enzymatic methods were: ESBM, SSBM, high solubles fish meal (**HSFM**), and low solubles fish meal (**LSFM**). The ESBM had been prepared by a process that reaches a maximum temperature of 163°C (Broderick, 1986); oil was extracted from SSBM at relatively low temperature using organic solvents, although some heat may have been applied in desolventizing the meal. These proteins were chosen because they have been tested extensively in our laboratory using in vivo studies and the IIV method (Broderick, 1986, 1992; Broderick et al., 1990). Results obtained from incubations with the enzyme mixtures were compared with those obtained from previous incubations of the same feedstuffs using the IIV method.

Incubations with *S. griseus* enzyme (Sigma Chemical, St. Louis, MO) were performed with 250 mg of each feed in 50-mL plastic tubes. One hour before the incubation, 20 mL of pre-warmed (39°C) borate-phosphate buffer was added to each tube, and tubes were held in a warm room at 39°C. A solution of .33 enzyme unit (**EU**)/mL of *S. griseus* protease was prepared in the borate-phosphate buffer and 5 mL was added to each tube, giving a final enzyme concentration of .066 EU/mL (Krishnamoorthy, 1982). Feeds were incubated in duplicate at 39°C for 0, 1, 4, 8, and 24 h (Poos-Floyd et al., 1985). Incubations were stopped by placing the tubes in an ice bath for about 30 min then freezing at –20°C. When thawed, samples were filtered through Whatman # 54 filter paper using a vacuum manifold. Tubes were rinsed and the residue was washed four or five times with a total of 200 mL of warm (39°C) distilled water. Filter paper and residues were transferred to Kjeldahl tubes for total N determination using a copper digestion catalyst (Kjeltabs®; Tecator, Herndon, VA [AOAC, 1980]).

Incubations with ficin (Sigma Chemical) were conducted using 15 mg N of each feed in 50-mL plastic tubes. Two hours before the incubation, 10 mL of a pre-warmed (39°C) ruminal micromineral buffer, 1 mL of 1% (wt/vol) NaN_3 (an inhibitor of microbial growth), and 1 mL of .5% (wt/vol) Triton X-100 were added to each vessel (Poos-Floyd et al., 1985), and held at 39°C. A solution of .5 EU/mL of ficin protease was prepared in a borate-phosphate buffer and 10 mL was added to each tube; the final enzyme concentration was .227 EU/mL. Incubations were stopped by adding 2 mL of 80% vol/vol *t*-butyl alcohol and placing the tubes in an ice bath for about 30 min; vessels then were stored frozen at –20°C until filtered. Tubes were filtered and the filter paper and residues analyzed for total Kjeldahl N as described above.

In the third enzyme system, incubations were conducted with a mixture of trypsin, carboxypeptidase B, chymotrypsin, and carboxypeptidase A (**TBCA**; Sigma Chemical). The endopeptidase trypsin was used to hydrolyze proteins at lysyl or arginyl peptide bonds such that the resulting polypeptides have a lysyl or arginyl terminal carboxyl group; these peptides were further cleaved by carboxypeptidase B, an exopeptidase that is specific for carboxy-terminal lysines or arginines (Hansen et al., 1975). The endopeptidase chymotrypsin and the exopeptidase carboxypeptidase A were added to trypsin and carboxypeptidase B to increase the total proteolytic activity. For incubations with the TBCA mixture, 15 mg N from each feed was weighed into 50-mL plastic tubes. One hour before the incubation, 20 mL of a pre-warmed (39°C) McDougall's buffer was added to each vessel and held at 39°C. A solution of TBCA enzymes was prepared in McDougall's buffer and 5 mL was added to each vessel. Several preliminary incubations (results not shown) were conducted to arrive at a total proteolytic activity similar to that of ruminal fluid in the IIV method using casein, ESBM, and SSBM as substrates. The final enzyme concentrations used were 116.6, .5, 2.5, and .5 EU/mL for trypsin, carboxypeptidase B, chymotrypsin, and carboxypeptidase A, respectively. Incubations were stopped by placing the tubes in an ice bath for about 30 min then

freezing at −20°C. When thawed, samples were filtered and the filter paper and residues analyzed for total Kjeldahl N as described.

Rates and extents of degradation in the IIV method were determined as described by Broderick (1987). For the enzymes, the degradation rates were estimated from linear regressions of the natural logarithm of residual N on time. Fraction A was determined as total sample N minus N in the residue collected after wetting the proteins with the buffer solutions before incubation with the enzymes; fraction A was assumed to be completely degraded. Fraction B was determined as total sample N minus fraction A. Extent of protein degradation was estimated using the following equation:

$$\text{Degraded protein (}\mathbf{DP}\text{), \%} = A + B\,(k_d/(k_d + k_p))$$

where k_d is the degradation rate and k_p, the ruminal passage rate, is assumed to be .06/h, as reported for lactating dairy cows (Hartnell and Satter, 1979).

Experiment 3. A set of 15 feedstuffs previously tested using the IIV technique were incubated with the TBCA enzyme mixture. Release of NH_3 N and TAA N were used in the computations for determining rate and extent of degradation using the SRF inoculum as described in the IIV method (Broderick, 1987). For the TBCA method, the incubations and computations for rate and extent of degradation were performed as described for the IIV method; because there was no NH_3 release, only the TAA N release was used in the computations.

Results obtained in Exp. 1 and 3 from incubating the *p*-nitroanilides or proteins with the enzyme mixtures and SRF, respectively, were compared using a two-tail, unequal variance *t*-test (Steel and Torrie, 1980). Fraction A and rate and extent of protein degradation obtained in Exp. 2 were analyzed using a one-way ANOVA by the GLM procedure of SAS (1985); protein was the main effect and treatment means were separated using a protected LSD ($P < .05$).

Results and Discussion

Experiment 1. Rates of *p*-nitroaniline release using SRF as inoculum indicated a wide range of activities toward the artificial substrates (Figure 1). These responses were assumed to be representative of the proteolytic activity in SRF. Brock et al. (1982) studied the proteolytic activity of whole ruminal fluid and concluded that ruminal bacteria possessed primarily serine, cysteine, and metalloproteinases, suggesting a broad range of specificities. Results obtained from incubating the artificial substrates with individual proteases are shown in Table 3. None of the individual enzyme sources possessed activity similar to that expressed by SRF. As expected, some enzymes had activity only toward a specific substrate, whereas others were active toward a range of substrates. Five enzyme sources had essentially zero activity toward all 13 substrates. The ficin solution became flocculent in the incubation medium and could not be assayed. This interference may have been caused by the ethanolic solution of the substrates. *Streptomyces griseus* protease, chymotrypsin, and Proteinase K were mixed at, respectively, .042, 2.5, and .5 EU/mL in the final medium, to give a composite activity similar to that of SRF. Although activity of the mixture toward the *p*-nitroanilides resembled that of SRF, activities were different ($P < .05$) from SRF for four of the 13 substrates (Figure 1), indicating that the mixture was not ideal. However, activities differed by only about 20% for two of the four substrates and by about 45% for a third; only activity toward L-

Table 3. Rate of *p*-nitroaniline release (nmol/[min·ml]) from 13 artificial substrates[a] after incubations with strained ruminal fluid (SRF) and commercial proteases as inoculum sources

	Artificial substrate (*p*-nitroanilide)												
Inoculum[b]	L-Val	L-Lys	L-Ala	L-Met	L-Leu	L-Pro	Gly	L-Arg	BTyr	BArg	BCys	L-Ala_2	L-Ala_3
SRF	2.4	4.6	3.0	4.5	6.6	3.9	2.4	5.2	1.6	10.4	2.5	5.4	3.1
A. oryzae	ND[c]	2.5	1.9	3.8	20.4	.2	3.8	1.5	.2	ND	.9	.7	ND
A. sojae	ND	ND	ND	.2	5.7	ND	ND	ND	ND	ND	ND	.3	ND
B. licheniformis	ND	ND	ND	ND	.2	ND	ND	ND	ND	ND	ND	.3	.4
B. polymyxa	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	.1	ND
Chymotrypsin	ND	ND	ND	ND	ND	ND	ND	ND	3.2	.2	ND	ND	ND
S. caespitosus	ND	.1	ND	.3	3.0	ND	ND	ND	.3	.8	.5	.4	ND
S. griseus	.04	.6	.4	2.0	7.8	.2	.1	.5	.3	14.0	1.1	.3	ND
Proteinase K	3.4	2.4	6.3	4.1	3.4	.3	1.9	2.1	.4	ND	2.4	1.9	2.8
Trypsin	ND	ND	ND	ND	ND	ND	ND	ND	ND	10.4	ND	.4	ND

[a]Artificial substrates identified according to the codes in Table 1.
[b]*B subtilis, B. thermoproteolyticus*, bromelain, elastin, and papain had no detectable activity and ficin formed a flocculent suspension in the incubation solution.
[c]Not detectable.

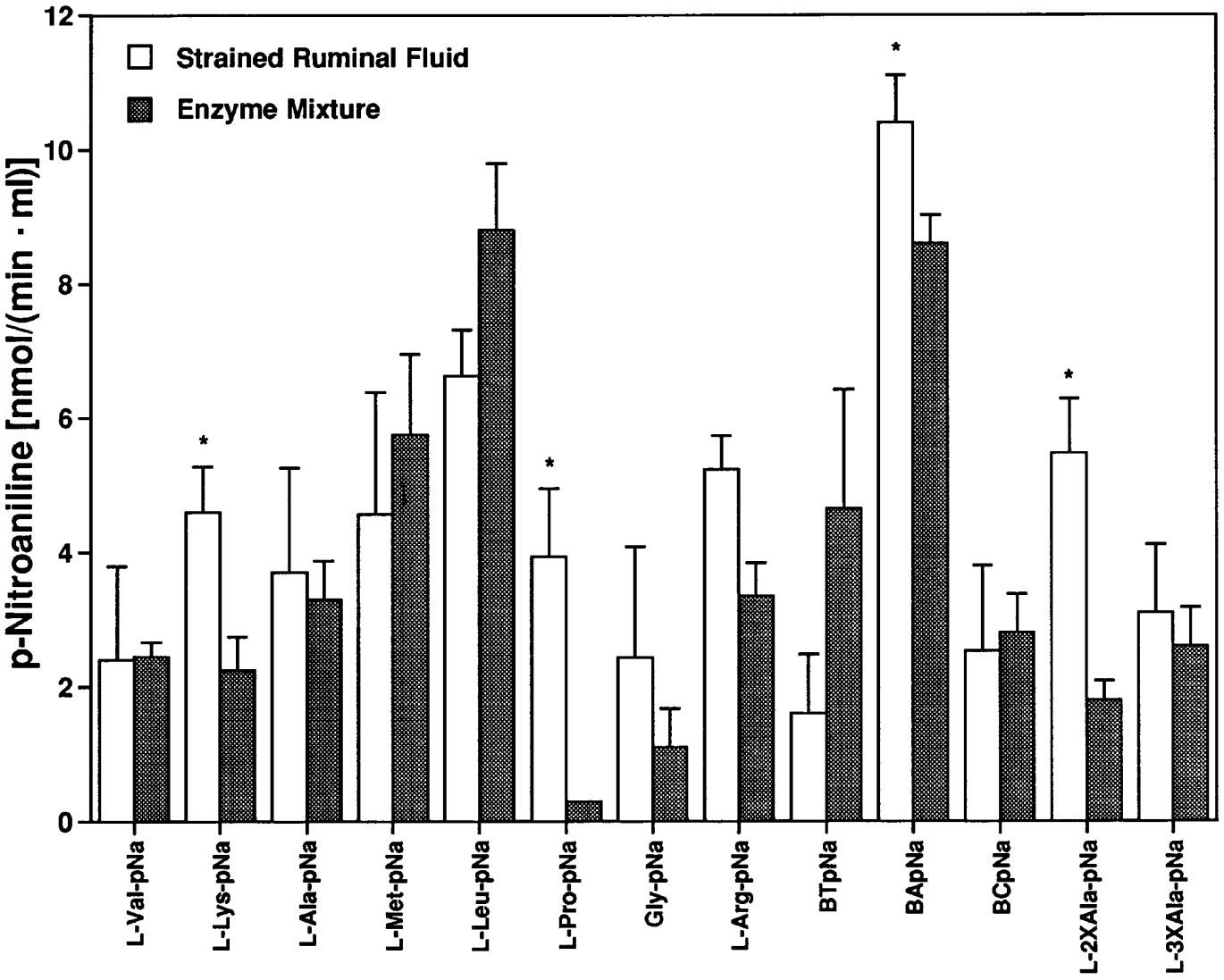

Figure 1. Rate of *p*-nitroaniline release (nmol/[min·mL]) from incubations of 13 artificial substrates with strained ruminal fluid or an enzyme mixture of *S. griseus*, chymotrypsin, and proteinase K at .042, 2.5, and .5 EU/mL of medium, respectively. Within a substrate, means with asterisks are different ($P < .05$). Vertical bars represent ± 1 SD.

proline *p*-nitroanilide was substantially lower in the enzyme mixture (Figure 1). Attempts were made to correct for these differences by adjusting concentrations of individual enzymes in this mixture. However, the activity toward other substrates also changed, resulting in even greater differences. Thus, the final protease mixture chosen was that depicted in Figure 1.

Results from incubations of casein, SSBM, and ESBM using the mixture of the three enzymes and SRF as inoculum sources are shown in Table 4. Degradation rates were slower with the enzyme mixture than with SRF with the exception of ESBM. Furthermore, the relative degradation rates found for ESBM and SSBM were opposite to those previously observed for the same proteins using the SRF inoculum and in vivo (Broderick, 1986). This indicated that the activity of the enzyme mixture toward feedstuff proteins differed from that with the artificial substrates. The reversal of activity toward ESBM and SSBM indicated that the enzyme mixture would be insensitive to differences resulting from heat treatment of proteins. Although the degradation rate of ESBM was relatively high, low degradation rates for casein and SSBM suggested that total proteolytic activity of the enzyme mixture was too low. These unexpected results may have arisen because 1) artificial substrates were not sensitive to differences in degradability among intact proteins because secondary and tertiary structure determines rate and extent of proteolysis of feed proteins or 2) a mixture of only three proteases is too simple and does not represent the much more complex, proteolytic attack by ruminal microorganisms where physical and chemi-

Table 4. Comparison of degradation rates (k_d) from incubations of casein, solvent soybean meal (SSBM), and expeller soybean meal (ESBM) using strained ruminal fluid (SRF)[a] or an enzyme mixture[b] as inoculum

Protein source	Degradation rate per hour			
	SRF	SD	Enzyme mixture	SD
Casein	.24	.050	.08	.004
SSBM	.15	.026	.05	.004
ESBM	.03	.007	.08	.001

[a]Means obtained during 1989 to 1992 were from 82, 68, and 73 incubations with, respectively, casein, SSBM, and ESBM using SRF as inoculum in the inhibitor in vitro method (Broderick, 1987).
[b]Mean values from 5, 3, and 2 incubations with casein, SSBM, and ESBM, respectively. The enzyme mixture consisted of *S griseus*, chymotrypsin, and Proteinase K at, respectively, .042, 2.5, and .5 EU/mL.

cal modifications of feeds may render proteins more or less susceptible to degradation (Wallace, 1994).

Experiment 2. Results obtained from incubations with the commercial enzymes and SRF are shown in Table 5. Generally, there were differences in the size of the fraction A estimated by the commercial enzyme methods; fraction A in SSBM and HSFM was larger than that in ESBM and LSFM (Table 5). The magnitude of fraction A, estimated using the commercial enzyme methods, was 10 to 30 times greater than that determined with the IIV technique. Differences in fraction A between the enzyme methods and the IIV technique were not unexpected. With each commercial enzyme, fraction A principally reflects solubility of CP in the buffer used with that particular enzyme. In the IIV method, the fraction A represents nonprotein N, measured as the sum of the net NH_3 plus TAA N present at time 0 and is perhaps a truer representation of CP degraded at 0 h. Fraction A is assumed to be completely degraded when computing DP. Moreover, Mahadevan et al. (1980) observed similar degradation rates for soluble and insoluble proteins isolated from SSBM. Although degradation rates were not different among the four proteins using the *S. griseus* protease, degradation rates for the two fish meals were slower than for the two soybean meals using ficin (Table 5). With TBCA, the degradation rate for ESBM actually was faster than for SSBM. Degradation rates for SSBM with SRF were two to three times more rapid than those obtained using ficin and TBCA. Despite the differences in fraction A and degradation rate, the *S. griseus* protease, ficin, and IIV methods ranked the four proteins similarly for extent of degradation.

When proteins are subjected to high temperatures, aldose sugars react with the amino acid residues of lysine or arginine, rendering them nutritionally unavailable (Hurrell and Finot, 1985). Thus, extent of hydrolysis by the TBCA mixture should be reduced for ESBM. However, differences in extent of degradation

Table 5. Fraction A[a], degradation rate (k_d), and extent of degradation of expeller soybean meal (ESBM), solvent soybean meal (SSBM), high solubles fish meal (HSFM), and low solubles fish meal (LSFM) in incubations using *S. griseus*[b], ficin[c], a mixture of trypsin, carboxypeptidase A, chymotrypsin and carboxypeptidase B (TBCA[d]), or strained ruminal fluid (SRF[e]) as inoculum

Feed protein	Fraction A, %				k_d, per hour				Extent of degradation, %			
	S. griseus	Ficin	TBCA	SRF	*S. griseus*	Ficin	TBCA	SRF	*S. griseus*	Ficin	TBCA	SRF
ESBM	21^{y}	18^{x}	12^{x}	1	.018	$.034^{w}$	$.086^{w}$	.030	39^{x}	47^{x}	64	31
SSBM	42^{w}	29^{w}	32^{w}	2	.024	$.045^{w}$	$.056^{x}$	.130	58^{w}	59^{w}	65	67
HSFM	37^{wx}	29^{w}	30^{w}	3	.020	$.015^{x}$	$.051^{y}$	.066	52^{w}	43^{y}	62	54
LSFM	26^{xy}	17^{x}	18^{wx}	2	.015	$.015^{x}$	$.054^{xy}$	.034	40^{x}	34^{z}	57	37
SEM	3.5	2.2	3.7	—	.004	.005	.002	...	2.9	1.0	3.2	...
$P > F$	.044	.029	.051	...	.381	.013	.001	...	.023	<.001	.424	...

[a]Percentage of N degraded at 0 h.
[b]*S. griseus* final enzyme concentration was .066 EU/mL incubation.
[c]Ficin final enzyme concentration was .227 EU/mL incubation.
[d]Trypsin, carboxypeptidase B, chymotrypsin, and carboxypeptidase A at final enzyme concentrations of 116.6, .5, 2.5, and .5 EU/mL incubation, respectively.
[e]Used as inoculum source with the inhibitor in vitro method (Broderick, 1987). Data for ESBM and SSBM are means from, respectively, 68 and 73 incubations, and data for HSFM and LSFM are from Broderick (1992).
[w,x,y,z]Means within the same column with different superscripts differ ($P < .05$).

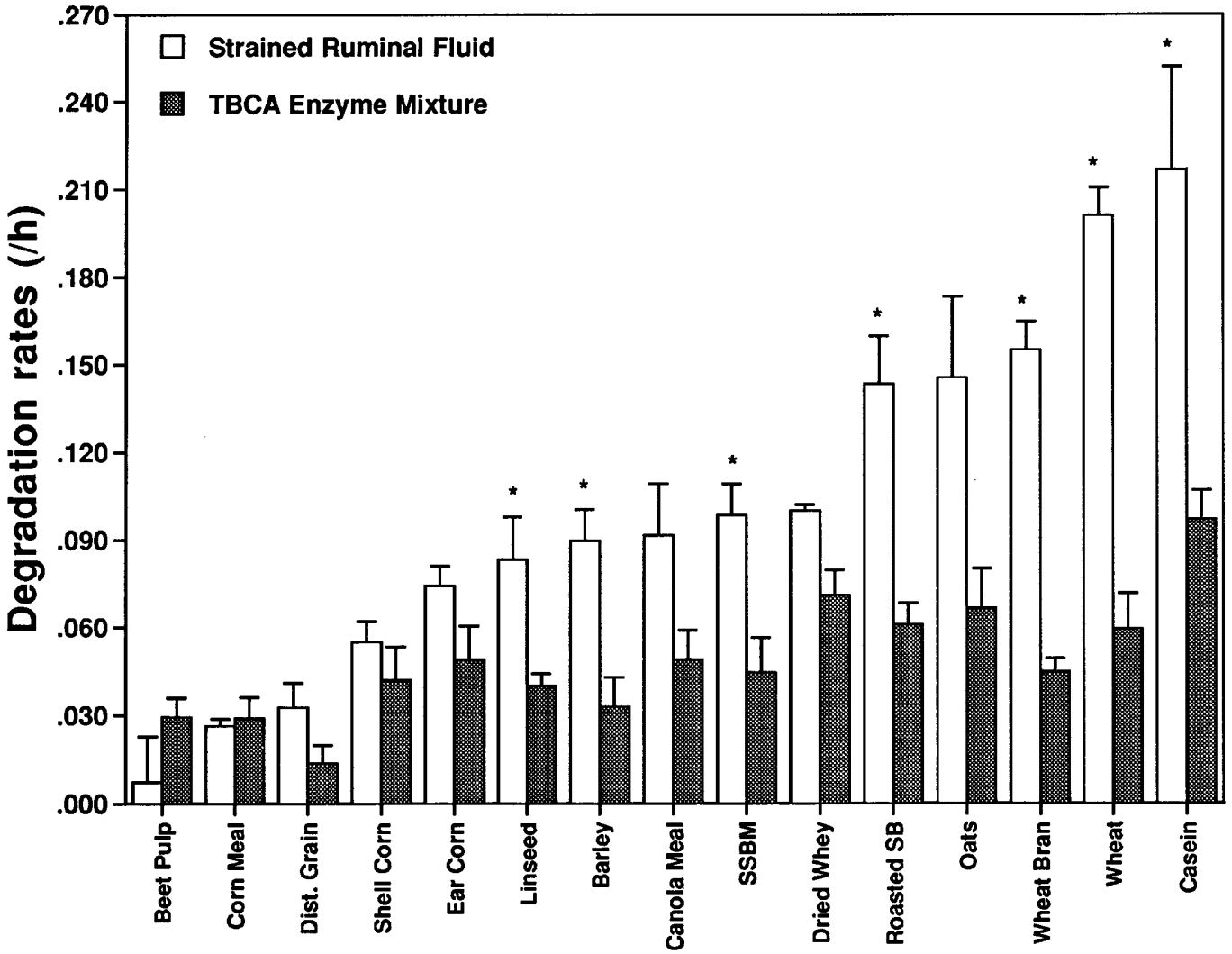

Figure 2. Degradation rates (per hour) determined in incubations of 15 feed proteins with strained ruminal fluid or a mixture of trypsin, carboxypeptidase B, chymotrypsin, and carboxypeptidase A (TBCA) at, respectively, 116.6, .5, 2.5, and .5 EU/mL. Within a protein, means with asterisks are different ($P < .05$). Vertical bars represent ± 1 SD.

were not detected between ESBM and SSBM, or among all four proteins, using the TBCA enzyme mixture (Table 5). This suggests that differences in the rate and extent of degradation obtained using SRF may be influenced by factors in addition to heat treatment. Discrepancies in the magnitude of fraction A and degradation rates among different methods have been reported by others (e.g., Broderick et al., 1988). When computed from these values, however, the extents of degradation were similar. For the enzyme systems, larger fractions A compensated for slower degradation rates for fractions B, resulting in similar extents of degradation.

Experiment 3. The TBCA enzyme mixture and IIV method using SRF were compared as the inocula in incubations with a set of 15 feed proteins. Methods for computing rate and extent of protein degradation in both procedures were the same as described earlier. Figure 2 depicts the degradation rates obtained in incubations with SRF and the TBCA enzyme mixture. Estimated degradation rates with TBCA ranged from .010 to .079/h and with SRF ranged from .007 to .217/h. Overall, degradation rates determined using the TBCA enzyme mixture as inoculum were slower ($P = .004$) than rates determined using SRF. Thus, it was concluded that the TBCA enzyme system was insensitive to substantial differences in ruminal degradability among typical feed proteins.

Implications

A mixture of three commercial proteases, formulated to have activity toward amino acid-*p*-nitroanilides similar to strained ruminal fluid, did not hydrolyze feed proteins at the same rate or extent as

strained ruminal fluid. Differences in fraction A and degradation rate of fraction B were found between strained ruminal fluid and two individual commercial proteases; however, the commercial proteases and strained ruminal fluid ranked proteins similarly for extent of degradation. Therefore, differences in fraction A and degradation rate compensate for each other. A mixture of four proteases (trypsin, carboxypeptidase B, chymotrypsin, and carboxypeptidase A) designed to detect extent of heat damage was insensitive to large differences in degradation rate. Because commercial proteases did not mimic the degradation rates obtained with strained ruminal fluid, commercial enzymes will prove unreliable for estimating rate and extent of ruminal protein degradation.

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