J. Dairy Sci. 94:3928–3935
doi:10.3168/jds.2010-3752

Contribution of exopeptidases to formation of nonprotein nitrogen during ensiling of alfalfa

L. Tao,*† H. Zhou,† X. S. Guo,*[1] R. J. Long,* Y. Zhu,† and W. Cheng‡
*MOE Key Laboratory of Arid and Grassland Ecology, School of Life Science, Lanzhou University, Lanzhou 730000, P. R. China
†Institute of Grassland Science, College of Animal Science and Technology, China Agricultural University, Beijing 100193, P. R. China
‡Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu 610041, P. R. China

ABSTRACT

The experiment was conducted to investigate the exopeptidase classes in alfalfa (*Medicago sativa* L.) leaves, and to determine their contribution to the formation of nonprotein nitrogen (NPN) components during ensiling. Six classes of inhibitors that included bestatin (aminopeptidase inhibitor), potato carboxypeptidase inhibitor (PCI, carboxypeptidase inhibitor), 1,10-phenanthroline (dipeptidase inhibitor), diprotin A (dipeptidyl-peptidase inhibitor), butabindide (tripeptidyl-peptidase inhibitor), and dipeptide Phe-Arg (peptidyl-dipeptidase inhibitor) were used. To determine the contribution of each exopeptidase to the formation of NPN products, aqueous extracts of fresh alfalfa were fermented to imitate the proteolytic process of ensiled alfalfa and to ensure that each class of exopeptidase inhibitor would have immediate contact with the proteases in the alfalfa extract. Five classes of exopeptidases; namely, aminopeptidase, carboxypeptidase, dipeptidase, dipeptidyl-peptidase, and tripeptidyl-peptidase, were shown to be present in alfalfa leaves, each playing a different role in alfalfa protein degradation. Aminopeptidase, carboxypeptidase, and dipeptidase were the main exopeptidases contributing to the formation of NH_3-N. Among the 5 exopeptidases, tripeptidyl-peptidase appeared to be the principal exopeptidase in hydrolyzing forage protein into peptides, whereas carboxypeptidase and dipeptidase appeared to be more important in contributing to the formation of amino acid-N. Dipeptidyl-peptidase and tripeptidyl-peptidase did not play a role in the formation of NH_3-N or amino acid-N. Dipeptidase, carboxypeptidase, and tripeptidyl-peptidase were the principal exopeptidases for hydrolyzing forage protein into NPN during ensilage, and treatment with a mixture of the 5 inhibitors reduced the total NPN concentration in the fermented alfalfa extract to about 45% of that in the control after 21 d of fermentation.

Received August 22, 2010.
Accepted April 25, 2011.
[1]Corresponding author: guoxsh07@lzu.edu.cn

Key words: alfalfa silage, exopeptidase, proteolysis, nonprotein nitrogen

INTRODUCTION

Extensive proteolysis in ensiled legume forages usually reduces protein quality for ruminants. During the process of ensiling, most of herbage proteins are degraded into oligopeptides, free amino acids (**FAA**), ammonia, and other forms of NPN (Oshima and McDonald, 1978). Most of the animal favorable protein fractions (Cornell System) within ensiled legumes are converted into NPN, and the total concentration of FAA is reduced extensively after alfalfa is ensiled (Guo et al., 2008). Usually, the large proportion of NPN within alfalfa silages is poorly synchronized with available energy-yielding substrates for protein synthesis by rumen microorganisms; therefore, silage proteins are utilized less efficiently by rumen microbes than is N from fresh or dried forages (Siddons et al., 1985; Givens and Rulquin, 2004). Proteolysis in the ensiled forage results mainly from plant proteolytic enzymes (Oshima and McDonald, 1978; McKersie, 1981; Heron et al., 1988). McKersie (1981) demonstrated the presence of at least 3 proteolytic enzymes in lucerne; that is, carboxypeptidase, aminopeptidase, and acid proteinase. Each differed in their pH and temperature optima, as well as their sensitivity to inhibitors. Jones et al. (1995) also studied the relative characteristics of crude enzyme extracts on protein degradation in alfalfa and red clover leaves.

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB, 1992), peptidases are divided into 2 classes; namely, exopeptidase and endopeptidase, based on their actions on substrates and their active sites, respectively. Exopeptidase includes 6 main peptidases: aminopeptidase (EC 3.4.11), carboxypeptidase (EC 3.4.16), dipeptidase (EC 3.4.13), dipeptidyl-peptidase (EC 3.4.14), tripeptidyl-peptidase (EC 3.4.14), and peptidyl-dipeptidase (EC 3.4.15). So far, the enzymes involved in proteolysis during alfalfa ensilage have not been systematically characterized. Few studies have

addressed the contributions of different exopeptidases and endopeptidases toward proteolysis and formation of NPN components in ensiled alfalfa forage. The aims of this study were to clarify the classes of exopeptidases that are involved in proteolysis within ensiled alfalfa, and to determine the contribution of these exopeptidases to the formation of different NPN compounds during ensiling.

MATERIALS AND METHODS

Plant Material

Medicago sativa L. (alfalfa) variety 'Algonquin' was grown in experimental plots at the College of Pastoral Agricultural Science and Technology, Yuzhong Campus, Lanzhou University, China (35°57′N, 104°2′E). Fresh leaves, obtained from second-cut, early-bloom, field-grown plants were cut from the petioles on June 20, 2009, placed in small bags, and immediately frozen in liquid N. The samples were taken to the laboratory, where they were stored at −80°C for up to 3 d.

Crude Enzyme Extraction and Determination of Exopeptidase Classes in Fresh Alfalfa Leaves

Alfalfa leaves (5 g) were ground in liquid N (1:4, wt/vol) into a powder by using a mortar and pestle. The powder was then suspended in 20 mL of 25 m*M* Tris-HCl, pH 7.5, containing 1% (wt/vol) insoluble polyvinylpolypyrrolidone and 0.1% (vol/vol) β-mercaptoethanol. Samples were centrifuged (10 min at 20,000 × *g*), and the supernatants were used directly for peptidase activity assays. For the analysis of exopeptidase activities, extracts were desalted at 4°C by application to Sephadex G-25 columns (Sigma-Aldrich, St. Louis, MO) equilibrated with 25 m*M* Tris-HCl [pH 7.5, containing 0.1% (vol/vol) β-mercaptoethanol; Feller et al., 1977]. To determine the classes of exopeptidase in the crude enzyme extracts, inhibitor treatments included a specific inhibitor within each of the 6 defined exopeptidase classes (Table 1; NC-IUBMB, 1992). These included (1) an aminopeptidase inhibitor (bestatin; Schaller et al., 1995; Desimone et al., 2000); (2) a carboxypeptidase inhibitor (potato carboxypeptidase inhibitor, **PCI**; Rees and Lipscomb, 1982); (3) a dipeptidase inhibitor (1,10-phenanthroline; Zhang et al., 2007); (4) a dipeptidyl-peptidase inhibitor (diprotin A; Umezawa et al., 1984); (5) a tripeptidyl-peptidases inhibitor (butabindide; Book et al., 2005; Rose et al., 1996); and (6) a peptidyl-dipeptidase inhibitor (dipeptide Phe-Arg; Dasarathy et al., 1989). The first 5 inhibitors were obtained from Sigma Chemical Co. (St. Louis, MO), and the last was obtained from Tocris Bioscience (Bristol, UK). After dissolving each of the 6 specific exopeptidase inhibitors in dimethyl sulfoxide, triplicate samples of the desalted extracts were incubated at room temperature for 0.5 h, together with each specific inhibitor. The final concentrations of each inhibitor in the mixed incubates were 0.05 m*M* (bestatin), 0.825 μ*M* (PCI), 10 m*M* (1,10-phenanthroline), 0.5 m*M* (diprotin A), 0.025 m*M* (butabindide), and 0.25 m*M* (dipeptide Phe-Arg; Table 1). Controls were prepared with dimethyl sulfoxide but without the addition of the specific exopeptidase inhibitors.

At the end of the incubation period, the residual aminopeptidase activity was determined using 2 m*M* L-Leu-*p*-nitroanilide as substrate in 100 m*M* Na-phosphate buffer, pH 7.0 (Feller et al., 1977). The residual carboxypeptidase activity was determined using 2 m*M* *N*-carbobenzoxy-L-Phe-L-alanine as substrate in 100 m*M* Na-acetate, pH 5.0 (Feller et al., 1977). The residual dipeptidase activity was determined using 12.5 m*M* Ala-Gly in 25 m*M* Tris-HCl buffer, pH 8.8 (Sopanen, 1976). The residual dipeptidyl-peptidase activity was measured using 100 μ*M* Lys-Pro-AMC in 50 m*M* 3-(*N*-morpholino)-propane sulfonic acid (pH 7.2), and 2 m*M* β-mercaptoethanol as substrate (Davy et al., 2000). The residual tripeptidyl-peptidase activity were determined using 25 μ*M* Ala-Ala-Phe-7-amido-4-methylcoumarin

Table 1. Peptidase inhibitors used to identify enzyme class in main peptidases and changes in proteolytic activity in crude enzyme extract of alfalfa after peptidase inhibition treatment

Inhibitor	Inhibitor class	Concentration	Proteolytic activity[1] (%)	SD
Control	—	0 mmol/L	100	—
Bestatin	Aminopeptidase	0.05 mmol/L	10.8	0.99
PCI[2]	Carboxypeptidase	0.825 μmol/L	22.6	1.11
1,10-Phenanthroline	Dipeptidase	10 mmol/L	50.6	4.93
Diprotin A	Dipeptidyl-peptidase	0.5 mmol/L	29.3	2.04
Butabindide	Tripeptidyl-peptidase	0.025 mmol/L	22.4	1.34
Dipeptide Phe-Arg	Peptidyl-dipeptidase	0.25 mmol/L	100	—

[1]Proteolytic activity was expressed as absorbance of the incubation samples at 570 nm directly, and the proteolytic activity in the fresh alfalfa extract (control) was considered as 100%.

[2]Potato carboxypeptidase inhibitor.

(AAF-AMC) as substrate in 15 m*M* Tris, pH 7.0, with 5 m*M* $MgCl_2$ (Balow et al., 1986). The residual peptidyl-dipeptidase activity was determined using 0.2 m*M* Hip-His-Leu as substrate in 0.1 *M* Hepes, pH 8.0, with 20 μ*M* lisinopril (Dasarathy et al., 1989). The above residual enzyme activities were determined as described by Fischer et al. (1998) with some modification. Briefly, 1 mL of substrate was mixed with 0.25 mL of diluted enzyme, and the mixtures were incubated at 37°C for 1 h. Undigested substrates were precipitated by adding 2 mL of cold TCA (final concentration: 5%, wt/vol) and letting stand for 30 min before centrifugation (5 min at 12,000 × *g*). The liberated α-amino groups in the supernatants were determined by the ninhydrin reaction (Friedman, 2004), with the absorbance set at 570 nm to express enzyme activity. The assay performed without any of the inhibitors served as the reference of initial activity.

Determination of Concentration of Each Inhibitor Required for Different Exopeptidases

The preliminary trial (above) demonstrated the existence of 5 exopeptidases in alfalfa leaves; namely, aminopeptidase, carboxypeptidase, dipeptidase, dipeptidyl-peptidase, and tripeptidyl-peptidase. Thereafter, we proceeded to determine the concentrations of the specific inhibitors required to inhibit each of the 5 exopeptidase classes. After an extensive survey of the published literature, several concentrations were prepared for each inhibitor (Table 2). Incubations were conducted following the same methods described above, and 3 replicates were tested for each inhibitor concentration.

Table 2. Effect of inhibitor concentrations on the proteolytic activity in crude enzyme extract of alfalfa

Inhibitor and concentration	Inhibitor class	Proteolytic activity[1]
Control		100[a]
Bestatin	Aminopeptidase	
0.001 m*M*		85.9[b]
0.01 m*M*		80.2[c]
0.05 m*M*		10.8[d]
0.07 m*M*		8.7[d]
0.1 m*M*		0.0[e]
SEM		10.1
Control		100[a]
PCI[2]	Carboxypeptidase	
0.00825 m*M*		17.8[b]
0.04125 m*M*		14.2[c]
0.0825 m*M*		12.0[d]
0.4125 m*M*		0.1[f]
0.825 m*M*		4.0[e]
SEM		8.2
Control		100[a]
1,10-phenanthroline	Dipeptidase	
10 m*M*		50.5[b]
15 m*M*		40.1[c]
20 m*M*		28.6[d]
25 m*M*		20.4[e]
50 m*M*		18.1[e]
SEM		6.7
Control		100[a]
Diprotin A	Dipeptidyl-peptidase	
0.1 m*M*		71.4[b]
0.5 m*M*		29.2[c]
1 m*M*		10.9[d]
2 m*M*		0.4[e]
3 m*M*		0.0[e]
SEM		9.2
Control		100[a]
Butabindide	Tripeptidyl-peptidase	
0.025 m*M*		87.5[b]
0.25 m*M*		22.4[c]
0.375 m*M*		4.7[d]
0.5 m*M*		1.2[de]
0.75 m*M*		0.0[e]
SEM		10.1

[a–f]Means within a column with different superscripts differ ($P < 0.05$).

[1]Proteolytic activity was express as absorbance of the incubation samples at 570 nm directly, and the proteolytic activity in the fresh alfalfa extract (control) was considered as 100%.

[2]Potato carboxypeptidase inhibitor.

Preparation of Aqueous Extracts of Whole-Crop Alfalfa and Experimental Treatments

A second cut of alfalfa was taken on June 30, 2009, from the same experimental plots described above, leaving a stubble of about 10 cm. Fresh forage samples were immediately taken to the laboratory and chopped into lengths of about 1.2 cm using a paper cutter. The chopped forage was homogenized in a blender with a volume of water that was equal to 4 times the weight of the fresh forage. The aqueous extract was filtered through 4 layers of cheesecloth and centrifuged (1,000 × *g*) for 1 min to homogenize the squeezed particles and the crude lysate in the green juice. The supernatant fraction was poured into a 1,000-mL beaker, which was placed within an ice box and glucose was added to a final concentration of 1% (wt/vol). The prepared extract was dispensed into 20-mL tubes with screw caps. Each extract sample was untreated (control) or treated with 0.1 m*M* bestatin (A), 0.413 m*M* PCI (B), 50 m*M* 1,10-phenanthrolin (C), 2 m*M* diprotin A (D), 0.5 m*M* butabindide (E), or a mixture of the 5 inhibitors together (A+B+C+D+E) to identify the role of the 5 exopeptidases in proteolysis. Thereafter, all of the tubes were sealed tightly and fermented at 30°C; fermentation of the extracts prepared from alfalfa relied on the action of epiphytic bacteria.

Analytical Procedures

A 10-mL sample of the fresh alfalfa extract was analyzed for total N concentration (AOAC, 1980) before treatment. Three replicate tubes from each treatment

were opened after 12 h or after 1, 2, 3, 5, 7, 14, and 21 d of fermentation to measure the pH of the extract. The tubes were then immediately resealed and frozen at −80°C pending subsequent analysis. A 2.5-mL aliquot of 25% (wt/vol) TCA was added to a 10-mL sample of the thawed fermented extract and was allowed to stand at room temperature for 1 h or was kept overnight at 4°C to precipitate the protein. The solution was then centrifuged at 18,000 × *g* for 15 min, and the supernatant liquid was analyzed for NH_3-N and free amino acid N (**AA-N**) (Broderick and Kang, 1980). Nonprotein N concentration was measured as described by Licitra et al. (1996) using a 5-mL sample of the supernatant liquid. Peptide-N concentration was determined by the increase in AA-N in the supernatant liquid, after digestion with 6 *N* HCl for 21 h at 105°C under an N_2 atmosphere. Volatile fatty acid, lactic acid (Porter, 1992), and water-soluble carbohydrate (**WSC**; Thomas, 1977) concentrations in the fermented extract were also determined.

Statistical Analysis

Data from the determination of exopeptidase classes and inhibiting concentrations of specific inhibitors for each kind of exopeptidase, and the fermentation products on d 21 were analyzed by one-way ANOVA. Control means were compared with treatment means using the Duncan test of the SPPS package (SPSS 17.0, SPSS Inc., Chicago, IL). Data obtained from fermented alfalfa green extract with different inhibitor treatments and sampling times were analyzed with ANOVA to test statistical significance of additives, time of ensiling, and the additive × time interaction using the GLM procedure of SPSS. When the *F*-test indicated a significant ($P < 0.05$) effect, means separations were conducted using a least significant difference test. A probability of $P < 0.05$ was used to denote significance unless otherwise indicated.

RESULTS

Exopeptidase Classes in Alfalfa and the Concentration Required for Each Specific Inhibitor To Be Effective

The reductions in activity of the crude enzyme extracts by the inhibitors are shown in Table 1 and demonstrate the presence of 5 classes of exopeptidase in the alfalfa leaves. The effects of different inhibitor concentrations on the activity of each peptidase are shown in Table 2. Aminopeptidase activity could be completely inhibited by 0.1 m*M* bestatin. Maximum inhibition was obtained with 0.413 m*M* PCI for carboxypeptidase, and maximum numerical inhibition with 50 m*M* 1,10-phenanthroline for depeptidase. Therefore, we concluded that 0.413 m*M* PCI and 50 m*M* 1,10-phenanthroline in the crude extract could inhibit carboxypeptidase and dipeptidase. Diprotin A and butabindide could inhibit dipeptidyl-peptidase and tripeptidyl-peptidase activities at concentrations of 2 and 0.5 m*M*, respectively, in the fresh alfalfa crude extract.

Fermentation Characteristics of Alfalfa Green Extract

No propionic acid was detected in any of the fermented alfalfa extracts, and low concentrations of butyric acid were observed in the control, bestatin, diprotin A, and butabindide inhibitor treatments (Table 3). The pH values of the control, bestatin, PCI, and butabindide were lower ($P < 0.05$) than those of 1,10-phenanthroline or the mixture. However, the lower pH treatments were not equal to each other. These low pH treatments were also more acidic ($P < 0.05$) than diprotin A. Responses for WSC were slightly different, with relatively low WSC for diprotin A. Both PCI and 1,10-phenanthroline increased the fermentation of alfalfa extracts, as indicated by greater concentration of lactic acid ($P < 0.05$), compared with the control and the alfalfa extracts treated with bestatin, diprotin A, and butabindide.

Effect of Exopeptidase Inhibitors on the Formation of NPN Constituents

Effects of peptidase inhibitors on the formation of NH_3-N are shown in Table 4. No NH_3-N was detected in the fermented alfalfa extract treated with the dipeptidase-specific inhibitor or the mixture of 5 inhibitors on any date. On d 21, NH_3-N concentrations in the fermented alfalfa extracts treated with bestatin and PCI were 33 and 53% of that in the control fermented extract. The concentrations of NH_3-N in diprotin A and butabindide treatments on d 21 were greater ($P < 0.05$) than observed for the control treatment (Table 4).

On d 21, FAA concentrations were lower ($P < 0.05$) in the fermented extracts treated with PCI, 1,10-phenanthroline, and with the mixture of 5 inhibitors than in the control and other 3 inhibitor treatments (Table 5). In contrast, treatments with bestatin, diprotin A, and butabindide did not suppress the production of AA-N compared with the control treatment. Comparisons of the AA-N concentrations within different treatments on d 21 of fermentation indicated that almost 30% of the total free AA-N was formed by carboxypeptidase and dipeptidase. The mixture of 5 inhibitors reduced the production of free AA-N during fermentation of alfalfa extract by approximately 73%.

Table 3. Concentrations of water-soluble carbohydrate (WSC, g/L) and fermentation products in fermented alfalfa extract on d 21 of fermentation (g/100 mL)

Inhibitor	Inhibitor class	pH	WSC	Lactic acid	Acetic acid	Propionic acid	Butyric acid
Control	—	4.4[c]	0.84[c]	0.25[c]	0.15[ab]	0.00	0.02[a]
Bestatin (A)	Aminopeptidase	4.0[d]	1.48[b]	0.46[b]	0.08[ab]	0.00	0.01[b]
PCI[1] (B)	Carboxypeptidase	3.7[e]	0.19[d]	1.01[a]	0.18[ab]	0.00	0.00[c]
1,10-Phenanthroline (C)	Dipeptidase	5.4[a]	4.36[a]	1.11[a]	0.07[bc]	0.00	0.00[c]
Diprotin A (D)	Dipeptidyl-peptidase	5.0[b]	1.70[b]	0.49[b]	0.07[bc]	0.00	0.02[a]
Butabindide (E)	Tripeptidyl-peptidase	3.4[f]	0.71[c]	0.32[bc]	0.10[bc]	0.00	0.01[b]
A + B + C + D + E	—	5.4[a]	4.39[a]	0.26[c]	0.00[c]	0.00	0.00[c]
SEM		0.172	0.376	0.089	0.015	0.000	0.002

[a–f]Means within a column with different superscripts differ ($P < 0.05$).

[1]Potato carboxypeptidase inhibitor.

The lowest peptide-N concentration observed on d 21 of fermentation was in the butabindide-treated alfalfa extract (Table 6). Over the first 3 d of fermentation, the peptide-N concentrations increased consistently, but further increases did not occur because peptides were degraded into FAA. The concentration of peptide-N in the extracts treated with the mixture of 5 inhibitors was greater ($P < 0.05$) than that in the control extract at the end of fermentation, suggesting that these peptidase inhibitors could effectively inhibit the peptide degradation to FAA. After 21 d of fermentation, concentrations of peptide-N in the extracts treated with bestatin, diprotin A, and butabindide were lower than that in the control extract ($P < 0.05$).

On d 21, all of the exopeptidase inhibitors decreased ($P < 0.05$) proteolysis during fermentation of the alfalfa extract compared with the control treatment (Table 7). Numerically, the lowest NPN concentrations were observed in the PCI and 1,10-phenanthroline-treated alfalfa extracts, which shows that carboxypeptidase and dipeptidase peptidase played a main role in degrading protein into NPN during proteolysis. After fermentation of 21 d, the NPN concentration in alfalfa extract treated with bestatin was the greatest among the inhibitor treatments, indicating that aminopeptidase is not a major peptidase in proteolysis compared with the other 4 exopeptidases. The total NPN concentration was decreased by the mixture of 5 exopeptidase inhibitors after 21 d of fermentation by 45%, relative to the control treatment.

DISCUSSION

Classes of Exopeptidases in Alfalfa and Inhibiting Concentration of Each Inhibitor

The peptidase classes vary in different plant species (Rooke and Hatfield, 2003). Previous studies suggested that carboxypeptidase and acid proteinase might be the principal enzymes hydrolyzing the proteins of ensiled alfalfa forage (McKersie and Buchanan-Smith, 1982; Guo et al., 2007). Studies characterizing exopeptidases in other plants have been well documented (Sopanen, 1976; Davy et al., 2000; Yang et al., 2004; Book et al., 2005; Kumada et al., 2007), whereas few studies have been conducted to characterize these exopeptidases in

Table 4. Concentrations of NH_3-N in the fermented alfalfa extract treated with different specific enzyme inhibitors (mg/g of total N)

Inhibitor	Inhibitor class	0 d	0.5 d	1 d	2 d	3 d	5 d	7 d	14 d	21 d
Control	—	0.0	0.0	0.0	0.0	0.0	0.0	0.0[b]	26.0[a]	26.1[c]
Bestatin (A)	Aminopeptidase	0.0	0.0	0.0	0.0	0.0	0.0	0.0[b]	8.4[b]	8.7[e]
PCI[1] (B)	Carboxypeptidase	0.0	0.0	0.0	0.0	0.0	0.0	0.0[b]	12.2[b]	13.7[d]
1,10-Phenanthroline (C)	Dipeptidase	0.0	0.0	0.0	0.0	0.0	0.0	0.0[b]	0.0[c]	0.0[f]
Diprotin A (D)	Dipeptidyl-peptidase	0.0	0.0	0.0	0.0	0.0	0.0	18.9[a]	28.6[a]	37.7[a]
Butabindide (E)	Tripeptidyl-peptidase	0.0	0.0	0.0	0.0	0.0	0.0	13.4[a]	24.3[a]	30.3[b]
A + B + C + D + E	—	0.0	0.0	0.0	0.0	0.0	0.0	0.0[b]	0.0[c]	0.0[f]
SEM		0.00	0.00	0.00	0.00	0.00	0.00	0.56	2.40	4.12
Significance of effect		$P <$								
Inhibitor		0.001								
Time		0.001								
Inhibitor × time		0.001								

[a–f]Means within a column with different superscripts differ ($P < 0.05$).

[1]Potato carboxypeptidase inhibitor.

Table 5. Concentrations of free amino acid N in the fermented alfalfa extract treated with different specific enzyme inhibitors (mg/g of total N)

Inhibitor	Inhibitor class	0 d	0.5 d	1 d	2 d	3 d	5 d	7 d	14 d	21 d
Control	—	28.0	53.4^{c}	56.6^{c}	94.1^{a}	160.4^{a}	168.7^{b}	246.0^{c}	321.1^{c}	352.9^{bc}
Bestatin (A)	Aminopeptidase	28.0	64.3^{b}	64.2^{b}	87.9^{b}	83.4^{e}	97.0^{d}	213.0^{d}	273.7^{d}	367.8^{b}
PCI[1] (B)	Carboxypeptidase	28.0	40.3^{e}	72.3^{a}	69.3^{d}	121.2^{b}	167.3^{b}	232.5^{c}	236.3^{e}	244.3^{d}
1,10-Phenanthroline (C)	Dipeptidase	28.0	42.1^{e}	45.5^{d}	67.6^{d}	49.7^{f}	86.9^{d}	161.9^{e}	219.8^{e}	258.4^{d}
Diprotin A (D)	Dipeptidyl-peptidase	28.0	68.0^{a}	59.0^{c}	79.1^{c}	110.3^{c}	144.2^{c}	290.4^{b}	346.9^{b}	341.8^{c}
Butabindide (E)	Tripeptidyl-peptidase	28.0	47.0^{d}	69.4^{a}	62.7^{e}	91.1^{d}	215.0^{a}	361.7^{a}	381.6^{a}	403.2^{a}
A + B + C + D + E	—	28.0	43.4^{e}	45.4^{e}	62.6^{e}	85.3^{e}	85.3^{d}	87.7^{f}	109.8^{f}	116.3^{e}
SEM		0.10	13.3	28.7	24.5	23.0	59.1	73.4	77.2	34.7
Significance of effect		$P <$								
Inhibitor		0.001								
Time		0.001								
Inhibitor × time		0.001								

[a–f]Means within a column with different superscripts differ ($P < 0.05$).

[1]Potato carboxypeptidase inhibitor.

alfalfa. The present study indicates that 5 exopeptidases (aminopeptidase, carboxypeptidase, dipeptidase, dipeptidyl-peptidase, and tripeptidyl peptidase) existed in alfalfa.

For the complete inhibition of aminopeptidase in alfalfa extract, a bestatin concentration of 0.1 m*M* was required; similar results were reported by Desimone et al. (2000) for barley (*Hordeum vulgare* L. 'Angora') leaves. The concentrations of inhibitors required for inhibiting activities of dipeptidase, dipeptidyl-peptidase, and tripeptidyl-peptidase in the alfalfa extracts were all greater than observed in previous reports (Sopanen, 1976; Davy et al., 2000; Book et al., 2005). The differences between inhibitor concentrations required to inhibit exopeptidases in our study relative to previous reports might be explained on the basis of the different forages used as fermentation substrates.

Effects of Different Inhibitors on Green Alfalfa Extract Fermentation

All of fermented alfalfa extracts were well preserved, because no propionic acid and little butyric acid were detected. In contrast, low concentrations of lactic acid and high concentrations of WSC in 1,10-phenanthroline–treated alfalfa extract suggest that the inhibitor restricted the fermentation of alfalfa extract. As reported by Nsereko et al. (1998) that lactic acid bacteria and probably other bacteria, which were present on the crop at ensiling, could have been inhibited by 1,10-phenanthroline.

Contribution of Exopeptidases to the Formation of NPN Constituents During Fermentation of Green Alfalfa Extract

Ammonia and amines are largely end-products of microbial activity rather than plant enzymes (Heron et al., 1986). In the present study, using 1,10-phenanthroline or the mixture of all 5 inhibitors (perhaps due to the action of 1,10-phenanthroline) resulted in complete suppression of ammonia formation in alfalfa extract. The results were probably due to the inhibition of microbial activity by 1,10-phenanthroline (Nsereko et al., 1998) and thus the fermentation of the alfalfa extract,

Table 6. Concentrations of peptide-N in the fermented alfalfa extract treated with different specific enzyme inhibitors (mg/g of total N)

Inhibitor	Inhibitor class	0 d	0.5 d	1 d	2 d	3 d	5 d	7 d	14 d	21 d
Control	—	209	253.5^{c}	327.8^{ab}	302.6^{bc}	348.7^{ab}	379.7^{b}	334.7^{a}	273.4^{cd}	275.3^{bc}
Bestatin (A)	Aminopeptidase	209	254.2^{c}	306.6^{bc}	297.2^{c}	338.2^{bc}	422.8^{a}	337.0^{a}	317.2^{b}	224.4^{cd}
PCI[1] (B)	Carboxypeptidase	209	260.1^{c}	268.1^{c}	383.6^{a}	369.2^{a}	353.9^{b}	295.2^{a}	291.9^{bc}	285.4^{b}
1,10-Phenanthroline (C)	Dipeptidase	209	319.0^{a}	334.3^{ab}	324.9^{b}	378.0^{a}	358.0^{b}	295.2^{a}	258.1^{d}	274.0^{bc}
Diprotin A (D)	Dipeptidyl-peptidase	209	227.2^{d}	298.9^{bc}	293.1^{c}	315.1^{cd}	360.4^{b}	238.6^{b}	211.5^{e}	208.7^{d}
Butabindide (E)	Tripeptidyl-peptidase	209	229.5^{d}	266.5^{c}	268.0^{d}	335.0^{bc}	217.6^{d}	126.7^{c}	133.0^{f}	133.5^{e}
A + B + C + D + E	—	209	280.3^{b}	338.7^{a}	321.8^{b}	301.4^{d}	310.4^{c}	301.6^{a}	350.5^{a}	353.4^{a}
SEM		0.56	31.3	18.9	22.2	44	32.9	54.7	37.4	53.1
Significance of effect		$P <$								
Inhibitor		0.001								
Time		0.001								
Inhibitor × time		0.001								

[a–f]Means within a column with different superscripts differ ($P < 0.05$).

[1]Potato carboxypeptidase inhibitor.

Table 7. Concentrations of NPN in the fermented alfalfa extract treated with different specific enzyme inhibitors (mg/g of total N)

Inhibitor	Inhibitor class	0 d	0.5 d	1 d	2 d	3 d	5 d	7 d	14 d	21 d
Control	—	237	306.8^{bcd}	384.3^{ab}	396.7^{ab}	509.1^{a}	548.4^{a}	580.6^{a}	620.5^{a}	654.2^{a}
Bestatin (A)	Aminopeptidase	237	318.5^{bc}	370.8^{ab}	385.0^{ab}	421.6^{b}	519.8^{ab}	550.0^{ab}	589.3^{a}	601.0^{b}
PCI[1] (B)	Carboxypeptidase	237	300.4^{cd}	340.44^{b}	452.9^{a}	490.4^{a}	521.2^{ab}	537.4^{bc}	540.4^{b}	543.4^{cd}
1,10-Phenanthroline (C)	Dipeptidase	237	361.1^{a}	379.9^{ab}	392.5^{ab}	421.0^{b}	444.8^{c}	457.1^{d}	472.9^{c}	532.4^{d}
Diprotin A (D)	Dipeptidyl-peptidase	237	295.2^{d}	357.9^{ab}	372.1^{ab}	425.4^{b}	504.6^{b}	547.9^{ab}	587.0^{a}	588.3^{bc}
Butabindide (E)	Tripeptidyl-peptidase	237	276.5^{e}	335.9^{b}	330.8^{c}	426.1^{b}	432.5^{c}	501.7^{c}	538.9^{b}	566.9^{bcd}
A + B + C + D + E	—	237	323.6^{b}	384.1^{a}	384.4^{ab}	386.6^{c}	395.7^{d}	389.2^{e}	460.3^{c}	469.7^{e}
SEM		0.19	33.8	17.3	52.2	29.8	43.8	37.5	62.4	48.2
Significance of effect		$P <$								
Inhibitor		0.001								
Time		0.001								
Inhibitor × time		0.001								

[a–e]Means within a column with different superscripts differ ($P < 0.05$).

[1]Potato carboxypeptidase inhibitor.

which can be demonstrated by high concentrations of WSC in treatments containing 1,10-phenanthroline. The reduction of NH_3-N in the bestatin- and PCI-treated alfalfa extracts indicated that aminopeptidase and carboxypeptidase also played important roles in the formation of NH_3-N during fermentation of ensiled forage, because the single amino acid residues released by these 2 exopeptidases provided the precursors for the formation of NH_3-N. However, dipeptidyl-peptidase and tripeptidyl-peptidase did not contribute to the formation of NH_3-N.

Results from this series of experiments suggest that dipeptidase and carboxypeptidase play an important role in the formation of AA-N during the ensiling of forage, but that dipeptidyl-peptidase, tripeptidyl-peptidase, and aminopeptidase do not. Carboxypeptidase acts at the free C-terminal end and liberates a single residue (Barrett et al., 2004), whereas tripeptidyl-peptidase acts at the free N-terminal end of polypeptide chains and liberates tripeptides (Barrett et al., 2004). On d 21 of fermentation, the lowest peptide-N concentration in the butabindide-treated alfalfa extract indicated that tripeptidyl-peptidase was the principal exopeptidase for degrading protein into peptides.

Treatments of bestatin and diprotin A, respectively, decreased NPN by about 16 and 13% of the control after 21 d of fermentation. Therefore, aminopeptidase and dipeptidyl-peptidase played a small role in the formation of NPN during proteolysis, which agrees with the previous findings (McKersie, 1981; Guo et al., 2007). In addition, reductions in NPN relative to control of 27 and 21% of by PCI and butabindide on d 21 indicated that the carboxypeptidases and tripeptidyl-peptidase also played a role in the formation of NPN. Previous studies suggested that the carboxypeptidase and acid proteinase might be the principal enzymes that hydrolyze proteins of ensiled forage, because both enzymes were comparatively stable during the ensiling period and were still active after 21 d of ensiling (McKersie and Buchanan-Smith, 1982; Guo et al., 2007). Results of the current study further confirmed that dipeptidase, carboxypeptidase, and tripeptidyl-peptidase were the main enzymes that hydrolyze the proteins of the ensiled forage. These findings might provide some useful information for commercial settings in developing proteolysis-inhibiting additives for ensiled forage.

CONCLUSIONS

Five exopeptidases, aminopeptidase, carboxypeptidase, dipeptidase, dipeptidyl-peptidase, and tripeptidyl-peptidase, were present in alfalfa leaves, and each of them played a different role in alfalfa protein degradation and formation of different N constituents during fermentation of alfalfa green extract. Dipeptidase, carboxypeptidase, and tripeptidyl-peptidase were the principal exopeptidases contributing to proteolysis in ensiled alfalfa.

ACKNOWLEDGMENTS

Financial support from the National Natural Science Foundation of China (30800800) and the PhD Programs Foundation of Ministry of Education of China (200807301008) is gratefully acknowledged.

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