Short communication: Characteristics of proteolytic activities of endo- and exopeptidases in alfalfa herbage and their implications for proteolysis in silage

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ABSTRACT

The pH optimum and thermostability of both exopeptidases and endopeptidases were investigated in this study to elucidate the possible role of plant proteases in proteolysis during ensiling of alfalfa herbage. Proteolytic activities of 4 classes of endopeptidases (i.e., serine, metallo, aspartic, and cysteine peptidase) and 5 classes of exopeptidases (i.e., aminopeptidase, carboxypeptidase, dipeptidase, dipeptidyl-peptidase, and tripeptidyl-peptidase) were examined within pH values of 3 to 9, and within temperatures from 20 to 90°C. Serine and metalloproteases, the principal endopeptidases that hydrolyzed most of the protein to nonprotein nitrogen in alfalfa silage, had optimum activities at pH 4. Among the major exopeptidases contributing protein degradation in ensiled alfalfa, dipeptidase and tripeptidyl-peptidase had stable activities between pH 4 and 6, and carboxypeptidase activity was optimal at pH 5. The optimum temperature for most peptidase activities was 40°C. Proteolytic activities of both endo- and exopeptidases increased with the elevation of incubating temperature from 20 to 40°C. The pH value in well-preserved alfalfa silage is often above 4.0, and the temperatures in the ensiled mass range from 25 to 40°C. Therefore, high proteolytic activities between pH 4 and 6 and the temperature range of ensiled alfalfa suggest that plant peptidases play a role in hydrolyzing protein during prolonged storage.

Key words: exopeptidase, endopeptidase, alfalfa, proteolysis

Short Communication

Plant enzymes are usually thought to be responsible for most of the proteolysis of ensiled forage, with

subsequent degradation of AA attributed mainly to microbial activity (Heron et al., 1989). Kung and Bedrosian (2010) suggested that the contribution of plant proteases to nitrogenous changes during prolonged storage is doubtful because plant enzymes are quickly inactivated with a decrease in pH. However, the extent of proteolysis in ensiled forage could be affected by the pH optimum of plant enzymes, rate and extent of pH decreases (McKersie, 1985), plant species (Papadopoulos and McKersie, 1983), DM content (Muck, 1987), and temperature (Muck and Dickerson, 1988). Optimal pH values for plant enzymes are often varied in different crops. The acid and alkaline endopeptidases in corn leaves were reported to have optimum activity at pH 5.4 and 7.5, respectively (Feller et al., 1977). The overall pH optimum for ryegrass proteases was 5.0 to 7.0 (Heron et al., 1989). McKersie (1981) reported that for alfalfa, the optimum pH values were 4.5 for acid proteinase, 6.7 for neutral proteinase, 5.4 for carboxypeptidase, and 6.5 for amino peptidase. These studies suggest that the optimum pH of alfalfa plant enzymes appears to be lower than other crops. Moreover, unlike corn or grass silages, the pH value in ensiled alfalfa forage is often above 4.0 (Owens et al., 1999; Schmidt et al., 2009), and even after 10 mo of storage, its pH value was reported to be between 4.3 and 4.6 (Kung et al., 2003).

Previous studies on the pH optimum of plant enzymes were investigated in terms of a general classification of proteolytic enzymes, such as acid, neutral, and alkaline proteases. Our studies showed that 4 classes of endopeptidases (i.e., serine, cysteine, aspartic, and metallopeptidases) and 5 classes of exopeptidase (i.e., aminopeptidase, carboxypeptidase, dipeptidase, dipeptidyl-, and tripeptidyl-peptidases) were present in alfalfa herbage, and each of them played a different role in alfalfa protein degradation and formation of different N constituents during fermentation of alfalfa green extract (Guo et al., 2011; Tao et al., 2011). However, to our knowledge, no study has been conducted

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to investigate the pH optimum and thermostability of these peptidases. Thus, our objective was to clarify the pH optimum and thermostability of both exopeptidases and endopeptidases in alfalfa herbage, and to elucidate the possible role of plant enzymes in proteolysis during ensiling of alfalfa.

Alfalfa (*Medicago sativa* L. 'Algonquin') was grown at experimental plots of the China Agricultural University (116°18' E, 39°57' N) in Beijing, China. Fresh leaves were obtained from each of 4 experimental plots at the late budding stage, placed in small bags, and immediately frozen in liquid N. The samples were then taken to the laboratory, where they were stored at -80°C for later analysis.

Alfalfa leaves (5 g) from each field plot were ground in liquid nitrogen (1:4, wt/vol) into powder in a mortar and pestle. The powder was then suspended in 20 mL of 25 mM Tris-HCl (pH 7.5) containing 1% (wt/vol) insoluble polyvinylpyrrolidone and 0.1% (vol/vol) β -mercaptoethanol. Samples were centrifuged (10 min at 20,000 $\times g$), and the supernatants were used directly for peptidase activity assays. For the analysis of peptidase activities, extracts were desalted at 4°C by application to Sephadex G-25 columns (Huarui Co., Beijing, China) equilibrated with 25 mM Tris-HCl [pH 7.5, containing 0.1% (vol/vol) β -mercaptoethanol; Feller et al., 1977].

A substrate solution of Pro-Thr-Glu-Phe-(NO₂-Phe)-Arg-Leu for aspartic protease was prepared according to Cruz de Carvalho et al. (2001). Substrate of serine protease of N-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide was prepared as described by Wang et al. (2007). Substrate solution of cysteine proteases was prepared as described by Asano et al. (1999) with some modification. Briefly, 4-methylcoumaryl-7-amide (Z-Phe-Arg-MCA; same as Z-Phe-Arg-AMC) was dissolved in a minimal volume of dimethyl sulfoxide and then prepared in a 50 mM sodium acetate buffer (pH 4.0), containing 200 mM NaCl and 10 mM 2-mercaptoethanol to a stock concentration of 40 μ M. As a substrate of metalloproteinase, 2,4-dinitrophenyl (DNP)-Gly-Gly-Met-Arg was prepared according to Belozersky et al. (1990). Substrates for aminopeptidase, carboxypeptidase, dipeptidase, dipeptidyl-peptidase, and tripeptidyl-peptidase were prepared as previously described by Tao et al. (2011).

All enzyme activities were determined as described by Fischer et al. (1998) with some modification. Briefly, 1 mL of substrate solution was mixed with 0.25 mL of crude enzyme extracts of alfalfa leaves from each of the 4 field plots, and 4 replicates of the mixtures from each plot were incubated at 37°C for 30 min to determine the protease activity as a function of pH; and when the protease activity as a function of temperature was

determined, the mixtures were incubated at the designated temperature for 30 min. Undigested substrates were precipitated by adding 2 mL of cold TCA (5%, wt/vol) and standing for 30 min before centrifugation (5 min at 12,000 $\times g$). The liberated α -amino groups in the supernatants were determined by the ninhydrin reaction (Friedman, 2004), and the concentration (mg/L) of the liberated AA was used to express enzyme activity. The assay performed without substrate served as the blank for each enzyme activity tested, and blank values subtracted from the tested values. The protease activity as a function of pH was determined using the specific substrate in buffers, which had adjusted pH values ranging from 3 to 9 in 1-unit intervals. The buffer was adjusted to the designated pH by the addition of HCl or NaOH (McKersie, 1981). At the end of the incubation period, aliquots of incubated enzyme were assayed as described above. The optimum temperature of each protease was studied using a constant temperature bath at temperatures ranging from 20 to 90°C in 10°C intervals. Supernatant of alfalfa leaves, which contained mixed proteases, was combined with each specific substrate as described in the substrate solution preparation. At the end of the incubation period, aliquots of incubated enzyme were assayed as described above.

Data on enzyme activity responses to different temperatures and pH values were subjected to ANOVA using the one-way ANOVA procedure of SPSS software (SPSS 17.0 for windows; SPSS Inc., Chicago, IL), and separate ANOVA were completed for pH and temperature. Polynomial contrasts were used to test linear and quadratic effects of increasing pH and temperature, respectively. A probability of $P < 0.05$ was used to denote significance unless otherwise indicated.

Responses of endo- and exopeptidases to pH values are shown in Table 1. Activities of serine, aspartic, and cysteine peptidase had linear and quadric ($P < 0.001$) responses to increasing pH. Metallopeptidase activity had a linear decrease with increasing pH value ($P < 0.001$). Serine and metallopeptidase activities were optimal at pH 4 over the pH range of 3 to 9. Serine peptidase was inactive above pH 8, whereas metallopeptidase was inactive above pH 7. Therefore, these 2 peptidases appear to be the acid proteinase as classified by McKersie (1981). In contrast, aspartic peptidase was optimal at pH 7, and was almost inactive at pH 5, which suggests that this peptidase is a neutral proteinase (McKersie, 1981). Cysteine peptidase was optimal at pH 6 and exhibited high activity between pH 6 and 8. The optimal pH values of serine and metallopeptidase activities indicate that these 2 classes of peptidases are probably the major endopeptidases in

Table 1. Responses of proteolytic activities of endopeptidases and exopeptidases to different pH values

Proteolytic activity ¹	Incubation pH							SE	P-value	
	3	4	5	6	7	8	9		Linear	Quadratic
Endopeptidase										
Serine peptidase	4.9	8.5	1.9	1.1	1.0	0.0	0.0	0.64	<0.001	<0.001
Metallopeptidase	3.9	5.9	5.3	0.8	0.0	0.0	0.0	0.55	<0.001	<0.623
Aspartic peptidase	0.0	0.0	0.1	4.5	4.9	1.9	0.4	0.45	<0.001	<0.001
Cysteine peptidase	0.0	0.1	1.9	3.9	3.6	3.6	0.0	0.37	<0.001	<0.001
Exopeptidase										
Dipeptidyl-peptidase	3.3	3.2	2.9	2.5	2.3	2.2	2.3	0.09	<0.001	<0.001
Tripeptidyl-peptidase	4.3	4.9	4.9	5.1	5.9	5.4	5.4	0.10	<0.001	<0.001
Dipeptidase	4.9	5.5	5.8	5.5	5.4	6.5	6.7	0.12	<0.001	<0.001
Carboxypeptidase	2.1	4.6	6.3	3.0	2.5	0.5	0.0	0.45	<0.001	<0.001
Aminopeptidase	1.8	3.2	3.6	12.4	9.7	8.7	6.7	0.80	<0.001	<0.001

¹The proteolytic activity was express as liberated AA concentration (mg/L) in the mixtures of substrate solution and crude enzyme extract after incubation at 37°C for 30 min.

proteolysis during ensiling and storage. However, the pH optimum of aspartic peptidase and its response to pH values suggest that its role in proteolysis during ensiling of alfalfa silage is not as important as serine and metallopeptidases, and its contribution to proteolysis during the prolonged storage of alfalfa silage is doubtful. Research on alfalfa silage ensiled at different DM contents (from 201 to 560 g/kg of DM), and with or without addition of inoculants, showed that within the prolonged storage period of 35 to 300 d, pH values of the ensiled forage ranged from 4.29 to 5.08 (Mader et al., 1985; Schmidt et al., 2009). Characteristics of pH optimum and responses of the 4 endopeptidases to different pH values indicated that, of the peptidases identified in this study, serine and metallopeptidases appear to be the most important endopeptidases in hydrolyzing protein during ensiling of alfalfa, which is in agreement with previous reports (Pichard et al., 2006; Guo et al., 2011).

Activities of all exopeptidases displayed linear and quadratic ($P < 0.001$) responses to increasing pH value (Table 1). The optimal pH values of carboxypeptidase and aminopeptidase activity were at pH 5 and 6, respectively. Similar results were reported by McKersie (1981) who found that these 2 enzymes displayed maximal activity at pH 5.4 and 6.5, respectively. Previous studies showed that carboxypeptidase retained above 40% of its original activity in alfalfa silage after 21 d of ensiling (McKersie and Buchanan-Smith, 1982; Guo et al., 2007). Aminopeptidase activity decreased substantially when the pH decreased below 5 in the current study, similar to the aminopeptidase activity in the study of McKersie and Buchanan-Smith (1982) where aminopeptidase was inactive in alfalfa after 5 d of ensiling. Activities of dipeptidase and tripeptidyl-peptidase, however, were relatively constant between pH 4 and

6. The above result provides supporting evidence that, among the 5 classes of exopeptidases, dipeptidase, carboxypeptidase, and tripeptidyl-peptidase were the principal exopeptidases for hydrolyzing forage protein into NPN during ensiling (Tao et al., 2011). Moreover, all 5 classes of exopeptidases and the endopeptidases of serine and metallopeptidase were still active at pH 3, which further emphasizes that even decreasing the pH value to below 4 does not necessarily prevent proteolysis caused mainly by alfalfa plant proteinases (Heron et al., 1989).

Except for aspartic peptidase, which had only a quadratic ($P < 0.001$) response, the rest of the endopeptidases had linear and quadratic ($P < 0.001$) responses to increasing temperature (Table 2). The optimum temperature for serine peptidase, metallopeptidase, and cysteine peptidase was 40°C in our study, whereas aspartic peptidase had its optimum temperature for activation at 60°C. Even at 70°C, this peptidase exhibited a relatively high activity, indicating thermal stability of the enzyme. Activities of all exopeptidases had linear and quadratic ($P < 0.001$) responses to increasing temperature (Table 2). In agreement with the result of McKersie (1981), the optimum temperature for carboxypeptidase and dipeptidase activity was 40°C, but for aminopeptidase, its activity remained relatively constant from 30 to 50°C. The optimal temperature for dipeptidyl-peptidase and tripeptidyl-peptidase was 50°C. These results suggest that the optimum temperature for most peptidase activities in alfalfa herbage is 40°C. In addition, proteolytic activities of both endo- and exopeptidase increased when the temperature of incubation rose from 20 to 40°C, which could explain the increased proteolysis reported to occur in ensiled alfalfa when the storage temperature increased from 15 to 35°C (Muck and Dickerson, 1988). Studies have re-

Table 2. Responses of proteolytic activities of endopeptidases and exopeptidases to different temperatures

Proteolytic activity ¹	Incubation temperature (°C)									P-value	
	20	30	40	50	60	70	80	90	SE	Linear	Quadratic
Endopeptidase											
Serine peptidase	2.5	3.6	6.8	3.9	2.8	0.0	0.0	0.0	0.47	<0.001	<0.001
Metallopeptidase	1.7	3.1	4.9	3.2	0.4	0.0	0.0	0.0	0.37	<0.001	<0.001
Aspartic peptidase	0.2	0.8	6.0	11.6	13.5	6.5	0.0	0.0	1.07	0.319	<0.001
Cysteine peptidase	1.1	2.0	2.8	2.7	0.0	0.0	0.0	0.0	0.24	<0.001	<0.001
Exopeptidase											
Dipeptidyl peptidase	1.0	1.9	2.8	9.4	4.1	3.1	0.0	0.0	0.66	<0.001	<0.001
Tripeptidyl peptidase	1.7	3.4	5.8	6.3	5.3	1.5	0.0	0.0	0.55	<0.001	<0.001
Dipeptidase	9.2	18.4	19.3	17.9	6.8	2.7	0.0	0.0	1.84	<0.001	<0.001
Carboxypeptidase	6.2	12.3	14.7	5.9	4.5	0.0	0.0	0.0	1.26	<0.001	<0.001
Aminopeptidase	7.1	12.3	12.4	12.2	4.5	0.0	0.0	0.0	1.27	<0.001	<0.001

¹The proteolytic activity was express as liberated AA concentration (mg/L) in the mixtures of substrate solution and crude enzyme extract after incubation at the given temperatures (20 to 90°C) for 30 min.

ported that the maximum temperature of alfalfa ensiled in large bales (Han et al., 2004), tower silos (Merchen and Satter, 1983), and bunker silos (Goesser et al., 2011) to be approximately 40°C after 2 to 3 wk of storage. Temperatures in well-preserved alfalfa silage appear to range between 25 and 40°C (Jaster and Moore, 1988) and coincide with most peptidase activity as reported in our present study.

In summary, endopeptidases and exopeptidases in the alfalfa herbage had different responses to pH and temperature. The optimum pH values for endopeptidase of serine, metallo, and cysteine peptidase and for exopeptidase of dipeptidase, carboxypeptidase, and tripeptidyl-peptidase appeared to further confirm that they are major contributors to nitrogenous changes during the ensiling of alfalfa herbage. In addition, high proteolytic activities between pH 3 and 6 of these peptidases may suggest that they could play a role in hydrolyzing proteins during prolonged storage.

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